# PHENOTYPIC, PHYSICO-CHEMICAL AND GENETIC RESPONSES OF MAIZE ASSOCIATED WITH RESISTANCE TO *FUSARIUM VERTICILLIOIDES*

by

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# SUMMARY

Maize (*Zea mays* L.) is integral to the Southern African diet and is a major component of animal feed products. *Fusarium verticillioides* is an ubiquitous fungal pathogen that contaminates maize grain and is the primary causal organism of Fusarium ear rot (FER) of maize. Grain contamination by *F. verticillioides* results in two key issues being kernel rot and mycotoxin, specifically fumonisins, contamination. Thus, grain yield and grain quality can be significantly impacted. Ingestion of fumonisin-contaminated grain has been associated with certain health implications in humans and animals such as oesophageal cancer and neural tube defects in humans as well as brain, heart and kidney defects in animals. Cultural and biological management strategies have been explored but despite some advantages for production, these remain inefficient for the management of FER and fumonisin accumulation in maize. Furthermore, there are no registered fungicides available for FER/fumonisin management. Plant resistance offers the most promising approach to managing FER/fumonisins that is both sustainable and environmentally sound. Resistance to *F. verticillioides* is, however, complex and requires a comprehensive understanding of the mechanisms that contribute to it.

Several factors mediate resistance at different levels of infection yet their relative importance and how they correlate to resistance is not well characterised. This study aimed to determine the importance of structural, physico-chemical and genetic traits for resistance. The correlation of these traits with specific infection indicators including FER severity, *F. verticillioides* growth and fumonisin accumulation in maize grain was also established. Maize inbred lines and commercial cultivars were inoculated with *F. verticillioides* and different characteristics were analysed. Structural characteristics included silk length, husk coverage, pericarp thickness and kernel hardness. The physico-chemical properties analysed in this study comprised pH, moisture content, total nitrogen and carbon as well as phenolic acids. Finally, reverse transcription quantitative PCR was used to evaluate the genetic response of maize grain to *F. verticillioides* using pathogenesis-related genes (*PR1* and *PR5*) and as well as *peroxidase* gene expression.

There were significant linear correlations between *F. verticillioides* growth and fumonisin accumulation in both trials. Significant fungal infection only occurred in one of the two localities (Vaalharts) evaluated. Multifactor analysis revealed no significant relationships between the levels of resistance (structural, physico-chemical and genetic) and infection indicators. However, partial least squares analysis revealed strong associations between individual characteristics and infection indicators. Significant relationships between the genetic response of *PR5* and *peroxidase* genes and infection indicators were determined. Furthermore, significant inverse associations between pH and carbon to nitrogen ratio and infection indicators were found.

This study highlighted the importance of physico-chemical and genetic response, in relation to well-established infection indicators commonly used to select resistant genotypes. Furthermore, this study has provided evidence that pH, C/N ratio and the expression of defence-related genes can be, used by breeders, as additional indicators of resistance to FER/fumonisins.

# OPSOMMING

Mielies (*Zea mays* L.) maak ‘n integrale deel uit van die Suid-Afrikaanse dieet en is ‘n belangrike komponent van voedsel produkte vir diere. Die alomteenwoordige patogeen, *Fusarium verticillioides,* kontamineer mielie graan en is die primêre veroorsakende patogeen van Fusarium kopvrot (FKV) van mielies. Graan kontaminasie deur *F. verticillioides* veroorsaak twee belangrike kwessies naamlik kern vrot en mikotoksien (spesifiek fumonisiene) kontaminasie. Sodoende word beide die graan opbrengs en -kwaliteit beïnvloed. Die inname van fumonisien gekontamineerde graan was al geassosieer met sekere gesondheid implikasies onderskeidelik in mense, soos esofageale kanker en neurale buisdefekte, en brein-, hart- en nier defekte in diere. Verbouïngspraktyke en biologiese beheer strategieë was al ondersoek, maar ten spyte van sekere voordele vir produksie, bly hierdie metodes onvoldoende vir die beheer van FKV en die opbouing van fumonisiene in mielies. Daarbenewens, is daar geen geregistreerde swamdoders beskikbaar vir FKV/fumonisien beheer nie. Plant weerstand bied die mees belowende benadering om FKV/fumonisien te beheer, wat beide volhoubaar en omgewingsvriendelik is. Nietemin is die weerstand van *F. verticillioides* kompleks en benodig ‘n omvattende begrip van die meganismes wat bydra tot dit.

Verskeie faktore bemiddel weerstand by verskillende vlakke van infeksie, maar hul relatiewe belangrikheid en hul verwantskap tot weerstand is nog nie goed beskryf nie. Hierdie studie se doel was om die belangrikheid van die strukturele, fisio-chemiese en genetiese kenmerke van weerstand te bepaal. Die verwantskap van hierdie kenmerke met spesifieke siekte aanwysers, insluitend die erns van FKV, *F. verticillioides* groei en fumonisien akkumilasie in mielie graan was ook bevestig. Mielie teellyne en kommersiële kultivars was geïnokuleer met *F. verticillioides* en gevolglik was verskillende faktore geanaliseer. Strukturele eienskappe soos lengte, skaal dekking, perikarp dikte en kern hardheid was ingesluit. Die fisio-chemiese eienskappe sluit die pH, voginhoud, totale stikstof en koolstof sowel as fenoliese sure in. Verder was omgekeerde transkriptoom kwantitatiewe PKR gebruik om die genetiese reaksie van mielie graan tot *F. verticillioides* te bepaal, met die gebruik van patogenese-verwante gene (*PR1* en *PR5*) asook as peroksidase geen uitdrukking.

Daar was beduidende lineêre verwantskappe tussen *F. verticillioides* groei en fumonisien ophoping in beide van die proewe. Beduidende siekte ontwikkeling het net plaasgevind in een (Vaalharts) van die twee lokaliteite wat geevalueer was. Multifaktor analise het gewys dat geen beduidende verwantskappe tussen die groepe van weerstand indikators (struktureel, fisio-chemies en geneties) en infeksie aanwysers was nie. Nietemin, het “partial least squares’’ analise ’n sterk assosiasie getoon tussen individuele karaktereienskappe en infeksie aanwysers. Beduidende verwantskappe tussen die genetiese reaksie van *PR5* en

peroksidase gene en infeksie parameters was bepaal. Ten einde laaste was daar beduidende negatiewe assosiasies tussen pH en koolstof tot stikstof infeksie aanwysers gevind.

Hierdie studie het lig gewerp op die belangrikheid van fisio-chemiese en genetiese reaksie, in verwantskap tot bestaande infeksie aanwysers wat algemeen gebruik word vir die seleksie van weerstandbiedende genotipes. Die studie verskaf ook bewyse dat pH, C/N verhouding en die geen uidrukking van vededigingsverwante gene kan deur plantetelers gebruik word, as addisionele aanwysers van weerstand tot FKV/fumonisiene.

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# CONTENTS

# CHAPTER 1

## Phenotypic, physico-chemical and genetic responses of maize associated with resistance to *Fusarium verticillioides*: A review

### INTRODUCTION

Maize is the most important food crop in Southern Africa, with South Africa being the largest producer of maize within this region (Statistica, 2018). Maize yield can be hindered by several biotic and abiotic factors. This includes insect pests, diseases and harsh environmental conditions. Fusarium ear rot (FER) is a prevalent disease of maize in South Africa and is predominantly caused by the fungal pathogen *Fusarium verticillioides* (Saccardo) Nirenberg and, to a lesser extent, *F. proliferatum* (Matsushima) Nirenberg, *F. subglutinans* (Saccardo) Nirenberg and *F. temperatum* (Scauflaire and Munaut) (Schoeman *et al*., 2018). FER is a major constraint to maize production causing reduced yield and quality within both commercial and subsistence farming. The issues associated with this disease is two-fold in that *F. verticillioides* is able to cause maize kernel rot as well as produce a range of toxic secondary metabolites, mycotoxins, of which fumonisins are the most important. Fumonisins have been associated with major health implications in humans and animals including oesophageal cancer in humans, neural tube defects in new-born humans and leukoencephalomalacia in horses (Bucci *et al*., 1998; Shephard *et al*., 2007; Venancio *et al*., 2014).

Management options for FER is limited with cultural practices providing little protection against *F. verticillioides* (Munkvold *et al*., 2003a, Mazzoni *et al*., 2010). Furthermore, there are no registered fungicides available for the control of *F. verticillioides*. Improved host-plant resistance, therefore, provides the most feasible method for managing FER and fumonisin accumulation (Clements *et al*., 2004). There are no cultivars available that are immune to *F. verticillioides* and fumonisin contamination, however, studies have identified cultivars and inbred lines that display varying degrees of resistance (Janse van Rensburg *et al*., 2015; Rose *et al*., 2017).

Resistance of maize to FER and fumonisin contamination is quantitative. A number of factors have been documented to contribute to resistance within maize inbred lines (Picot *et al*., 2010), however, their relative importance in commercial cultivars and how they correlate to host-plant resistance is not well characterised. An understanding of how different factors of the crop contribute to resistance could help breeders select material more efficiently and help producers select cultivars more effectively. The aim of the review is to give an account of the

host defence mechanisms associated with the *F. verticillioides*-maize pathosystem. Particular focus will be placed on the role of structural, physico-chemical and genetic mechanisms of maize that potentially contribute to resistance to *F. verticillioides* and its fumonisins.

## MAIZE, A STAPLE FOOD AND LIVESTOCK FEED FOR SOUTH AFRICA

### The maize plant

Maize (*Zea mays* L.) owes its popularity as a cereal crop to its high-yielding ability. Maize has a diverse genetic background and, therefore, is adaptable to different agro-ecosystems (Krishna, 2012). It is an annual, monocotyledonous, caryopsis crop and its lifecycle can be divided into a vegetative stage and reproductive stage (Sheridan and Clark, 1994). During the vegetative growth stage, the plant develops an adventitious root system to scavenge for water and nutrients in the soil. The final root system can extend as far as 1.5 to 2 m into the soil. The plant also develops bract roots at the soil surface to further anchor the plant and scavenge the top layer of the soil towards the end of the vegetative growth stage (Fig. 1). The above-ground part eventually grows to about 1 to 4 m tall with the vegetative part consisting of an erect stem, nodes and internodes, as well as broad single leaves (Wusirika and Li, 2014). The mature plant usually has about 18 to 30 leaves, with one leaf at each node, with a cylindrical positioning although there is a lot of variation with regards to leaf number, size and orientation.

Once the plant has established the vegetative phase, it starts the reproductive phase during which much of its resources are translocated to the maize ears (Krishna, 2012). Maize is monoecious with the female inflorescence being the ears and the male inflorescence being the tassel (Cheng and Pareddy, 1994). The maize ear develops at the auxiliary bud apex (Fig. 1) and consists of a thick stalk with a number of ovaries (300 to 1000), each containing one ovule. At the base of each ovary is a point of silk attachment, where the silk elongates from and serves as receptive stigmas for pollination. The ear is enclosed by husk leaves (with silks extending out of it). The number, tightness and coverage of husk leaves vary greatly among different genotypes and even throughout the season (Krishna, 2012). Furthermore, the number, size and positioning of maize ears also differs among different genotypes. Tassels form at the apical meristem and are a branched structure. It contains anthers that produce pollen during anthesis (Cheng and Pareddy, 1994).

### Maize production

South Africa is a key role player in terms of maize production in Africa, with it being a crucial component in the diet of South Africans (FAO, 2012). South Africa is the ninth largest producer of maize in the world with a total area of 3 million hectares planted and total tonnage of 14.4

million produced in 2017/18 (FAO, 2012; DAFF, 2018). In most years, the country produces a surplus yield, which is then exported to surrounding African countries, with a smaller portion exported to Asian and European countries (BFAP, 2018; Ranum *et al*., 2014). Maize is a diverse crop with many applications due to its high nutritional value and adaptability to different growing conditions.

In South Africa, white maize constitutes 59% of the total maize production and yellow maize about 41% and is produced primarily for human consumption and animal feed, respectively (DAFF, 2018). Maize serves as a staple crop for marginalised, middle and upper-middle class consumers which make up to 80% of the adult population in South Africa (BFAP, 2018). Human consumption of maize in Africa is the highest in the world with a maximum consumption of 200 to 300 g per person per day in Southern Africa (FAO, 2012). Furthermore, in rural villages in South Africa maize consumption has been recorded as high as 700 g per person per day (Ranum *et al.*, 2014). Maize is mainly consumed as meal or flour; however, there are various industrial uses of maize including production of oils, sweeteners and starch used in canned and frozen food products as well as condiments (Singh *et al*., 2014). Furthermore, maize is also used to produce industrial alcohol, which in turn is used to produce ethanol and can be used as a biofuel additive (Ranum *et al*., 2014). Maize is also the main source of energy in animal feed. The percentage of maize produced for animal feed is expected to increase by 21% by the year 2027 as the demand thereof increases (BFAP, 2018).

Maize is a versatile crop grown in diverse environments. It is primarily produced under dryland conditions in South Africa, with 89% of the total maize produced in this manner (DAFF, 2018). Maize is also produced under irrigation in maize-growing regions (maize belt) where rainfall is typically lower. The South African maize belt includes the North-West, Free State, Gauteng, KwaZulu Natal, Mpumalanga and certain parts of Limpopo, Northern Cape and Eastern Cape. Most of the production is concentrated in the North-West, Free State and Mpumalanga, accounting for 83% of production in 2017/18 (DAFF, 2018).

South African maize production suffered greatly during the severe drought in the maize belt from 2015 to 2016 with production as low as 7.7 million tonnes (BFAP, 2018). This was especially severe in dryland maize production regions. As rainfall levels returned to normal the following season, production reached a record high of 16 million tonnes in the 2016/17 season (DAFF, 2018). This caused a decrease of 47% in the average cost of maize from the 2015/16 to the 2016/17 season (DAFF, 2018). With further increased production in the 2017/18 season, maize exports are greatly exceeding import estimates. Besides abiotic factors influencing the production of maize its further impacted by biotic stress conditions such as mycotoxigenic fungi that not only reduces grain yield, but also negatively affects humans and animals.

## FUSARIUM EAR ROT

Fusarium ear rot (FER) of maize poses a major constraint to the South African maize industry. FER is primarily caused by the mycotoxigenic fungus, *Fusarium verticillioides* and, to a lesser extent, *F. proliferatum*, *F. subglutinans* and, more recently, *F. temperatum* (Scauflaire and Munaut) (Scauflaire *et al.*, 2011). *Fusarium temperatum* is a newly described species that consists of isolates, previously identified as *F. subglutinans* but produces beauvericin, whereas *F. subglutinans* (as it is currently defined) only produces moniliformin (Moretti *et al*., 2008).

*Fusarium verticillioides* is the most frequently occurring pathogen species associated with maize worldwide and also South Africa (Oren *et al*., 2003; Boutigny *et al*., 2012; Schoeman *et al*., 2018). FER affects maize at all developmental stages causing yield losses and reduction of kernel quality (Munkvold and Desjardins, 1997; Small *et al*., 2012). *Fusarium verticillioides* causes rotting of maize kernels which is characterised by a white, light pink, starburst mycelial growth and degradation of kernel tissue occurring on random kernels, groups of kernels or damaged kernels (Koehler, 1942; Munkvold, 2003b; Small *et al*., 2012) (Fig. 2). The fungus is also able to produce toxic secondary metabolites known as mycotoxins. *Fusarium verticillioides* is able to produce several mycotoxins including fumonisins, fusarin, fusaric acid and moniliformin, however, fumonisins are considered the most important (Glenn, 2008). Infection by FER pathogens and mycotoxin contamination may occur with the visible rot symptoms or as an asymptomatic build-up in maize kernels (Gelderblom *et al*., 1988; Munkvold *et al*., 1999).

## *FUSARIUM VERTICILLIOIDES,* MYCOTOXIGENIC FUNGAL PATHOGEN OF MAIZE

### Biology and epidemiology

*Fusarium verticillioides* is a filamentous fungal pathogen that is able to produce sexual and asexual spores (Seo *et al.*, 2001). It is a heterothallic fungus with two mating types (MAT1-1 and MAT1-2) that forms perithecia upon sexual reproduction, resulting in genetic recombination (Li *et al*., 2006). Perithecia of *F. verticillioides* is not, however, as abundantly found in nature as that of other maize rot pathogens and is not a central component in the epidemiology of *F. verticillioides* (Munkvold, 2003a). The pathogen forms two kinds of asexual spores, microconidia and macroconidia, which are the main spore types for the spread and development of the disease (Li *et al*., 2006). Microconidia are single-celled spores that are found in chains on aerial mycelia and are the most abundant asexual spore type of the fungus (Nelson *et al*., 1994). Microconidia are airborne and are, therefore, pivotal to the spread of the

disease (Munkvold and Carlton, 1997). Macroconidia are larger, septated spores that are not as abundant as microconidia but still essential in the epidemiology and equally important to the characterisation of the fungus (Nelson *et al*., 1994). As the fungus sporulates profusely on crop residue, it can spread spores either via the wind or splashes of water (Munkvold and Desjardins, 1997). *Fusarium verticillioides* forms swollen hyphal cells that have thickened cell walls which act as pseudo-chlamydospores (Klaasen and Nelson, 1997; Fandohan *et al*., 2005).

*Fusarium verticillioides* can survive in soil and on crop residue (maize stubble) from the previous season, which subsequently serves as the primary source of inoculum for infection in the following growing season (Munkvold, 2003b) (Fig. 3). The pathogen is able to overwinter on maize stubble as mycelium or thickened hyphae (Klaasen and Nelson, 1997). Additionally, several alternative host crops and weed species have been identified to be a source of inoculum for *F. verticillioides* (Parry *et al.,* 1995). *Fusarium verticillioides* relies exclusively on wounds and natural openings for successful infection of the plant (Duncan and Howard, 2010). The fungus is not limited to infecting seedlings but can infect plants at all stages of development (Fandohan *et al.*, 2003; Venturini *et al*., 2011). *Fusarium verticillioides* can infect maize plants systemically from seed (Foley, 1962) and roots (Munkvold *et al.*, 1997), through wounds (Yates and Sparks, 2008) or via silks (Munkvold and Carlton, 1997) and is able to colonise virtually all parts of the plant (Oren *et al*., 2003). Infection through the silks is the most important pathway for the fungus as it provides access to the kernels (Munkvold *et al*., 1997; Cao *et al*., 2014). Silk infection also does not require the kernel to be damaged in any way and provides direct access to the kernel microenvironment (Cao *et al*., 2013).

When conidia lands on silk tissue, it is able to move down the silk channel in free water (Reid and Sinha, 1998; Duncan and Howard, 2010). It can then germinate and allow hyphae to grow on the cuticle until it reaches the base of the silk (Reid and Sinha, 1998). If the stylar canal is open at the base of the silk, it can be drawn into the open space and allow fungal hyphae to grow and directly gain access to the kernel microenvironment (Duncan and Howard, 2010; Mesterházy *et al*., 2012). Wounds can also serve as vital points of entry for the pathogen. Wounds can be due to insect feeding, animals, humans, machine damage or abrasions. Wounds can then be contaminated by airborne conidia or by contaminated water containing spores being splashed by rain or irrigation (Munkvold *et al*., 1997).

*Fusarium verticillioides* is a hemi-biotrophic pathogen. This means that the pathogen has an initial biotrophic lifestyle, therefore, can gain nutrients from living cells and can even colonise maize tissue asymptomatically but can also, under certain conditions, switch to a necrotrophic form by feeding off the organic matter of dead cells (Bacon *et al*., 2008; Horbach *et al*., 2011).

Varying results have been obtained regarding the effectiveness of systemic infection from seed on successful kernel infection, disease development and fumonisin accumulation (Munkvold *et al*., 1997; Oren *et al.,* 2003; Murillo-Williams and Munkvold, 2008). Differences can be attributed to several factors such as genetic, environmental and experimental design differences. However, there is a general consensus that systemic infection can lead to kernel infection. *Fusarium verticillioides* can be present inside or on the surface of the seed as mycelia or as a spore to infect the seedling as it develops (Munkvold *et al*., 1997). The subsequent systemic growth of the fungus as the plant develops can lead to root, shoot, stalk and ear rot (Munkvold *et al*., 1997; Li *et al*., 2006). Timing of infection is a key aspect for the fungus to establish disease progression and mycotoxin deposition within the plant (Reid *et al.*, 2002). Prevailing environmental conditions around the time of infection is paramount to successful or unsuccessful infection. In terms of infection through the silks, the silking period (anthesis) is a crucial time for infection while rain, temperature and relative humidity around silking are prominent aspects to consider (Marín *et al*., 2004). Moderate rain at silking causes splashes of water which could spread inoculum (Marín *et al*., 2004) while drier conditions during anthesis puts the plant under water stress which could make it more susceptible to fungal and insect attack (Marín *et al*., 2004). Environmental conditions that favour insect feeding could also increase the infection rate of *F. verticillioides* with insects acting as vectors for the fungus (Sobek and Munkvold, 1999). Elevated temperatures (between 25ºC to 30ºC) increase the rate of infection and sporulation of the fungus (Cao *et al*., 2014). Furthermore, with sufficient inoculum pressure and environmental conditions, infection can successfully take place at any stage of development.

**Fumonisins**

Fumonisins are the most important mycotoxin produced by *F. verticillioides* (Gelderblom *et al.*, 1988). It is a family of mycotoxins that comprises of four major groups, A, B, C, P with group B (FB) being the most abundantly found in nature (Alberts *et al*., 2016). Fumonisins contains a range of isoforms; however, fumonisin $B_1$ ($FB_1$) is the most abundant isoform when compared to the less oxygenated isoforms, including fumonisins $B_2$ ($FB_2$), $B_3$ ($FB_3$) and $B_4$ ($FB_4$) (Nelson *et al*., 1994; Proctor *et al*., 2002).

*Impact of fumonisins on human and animal heath*

Fumonisins are known to be associated with a number of human and animal health issues (Da Rocha *et al.*, 2014). Consumption of fumonisin-contaminated grain or grain products are associated with disruption of sphingolipid biosynthesis, and, therefore, lead to neural and cell signalling abnormalities (Norred *et al*., 1992; Brown and London, 2000). Fumonisins

competitively inhibits the synthesis of ceramide which disrupts the metabolism of sphingolipids that help to regulate apoptosis (Giorni *et al*., 2015; Blacutt *et al*., 2017). Wang *et al*. (1996) showed that the disruption of this pathway by fumonisins can lead to irregular cell death in mammalian kidney cells. Furthermore, a positive correlation was found between the regulation of mitosis and programmed cell death (PCD) (Hartwell and Kastan, 1994). Due to this association, it has been suggested that disruption of these pathways could lead to overexpression of PCD or proliferation of cells causing lesions and/or carcinogenesis (Wang *et al*., 1996).

Various studies have reported the association of fumonisins with human health issues (Reeder *et al*., 1992; Van Egmond *et al*., 2007; Da Rocha *et al*., 2014). Rheeder *et al*. (1992) compared maize samples from areas with high and low incidences of oesophageal cancer and found that areas with high incidences of oesophageal cancer had maize containing high levels of *F. verticillioides*, with visible and non-visible symptoms. Moreover, cases of fumonisins possibly leading to oesophageal cancer in humans have been noted in southern Africa, China and Italy (Da Rocha *et al*., 2014). Additional studies suggest that fumonisins are carcinogenic (Riley *et al*., 1994; Gelderblom *et al*., 2002; Rheeder *et al*., 2002; Van Egmond *et al*., 2007). Fumonisins have been suggested to cause neural tube defects (NTD) and craniofacial abnormalities in new born humans (Marasas *et al*., 2004). In a study by Hendricks (1999), increased levels of NTD incidence was found along the Texan-Mexican border which was related to a high consumption of homemade tortillas during the first trimester of pregnancy as opposed to store-bought tortillas.

Mycotoxins in animal feed are an important yet complex issue. The ingestion of mycotoxin-contaminated feed does not always result in death but rather leads to reduced productivity, vitality, loss of weight or damage to certain vital organs (Varga and Tóth, 2005; Wu, 2007). Fumonisins seem to affect three major kinds of tissue in animals, that being neural, lung and liver tissue (Kellerman *et al*., 1990; Rheeder *et al*., 1992), however, fumonisin toxicity-related abnormalities are not limited to these related organs. Kellerman *et al*. (1990) showed that ingestion of fumonisins can cause leukoencephalomalacia in horses, which is a neurotoxic disease. In a study by Kriek *et al*. (1981a), the effect of fumonisins on different animal species was assessed. They found that in non-human primates, the effect of fumonisins was based on the dosage ingested, in that higher dosages caused heart failure whereas lower dosages caused liver damage (Kriek *et al*., 1981a). Bucci *et al*. (1998) looked at the effects of fumonisins in horses and also noted a dose-dependent effect of fumonisins. Higher dosages are related to neural defects, like brain lesions (causing leukoencephalomalacia) and lower dosages associated with renal toxicity, causing renal lesions (Bucci *et al*., 1998). Hashek *et al*. (1992) also demonstrated this dose-dependent effect of fumonisins in swine where lower dosages affects liver tissue and higher dosages causes acute pulmonary oedema and may

cause death. Fumonisins also affect the liver and kidney of sheep, however, the dose dependent effect was not noted in sheep (Kriek *et al*., 1981b).

*Legislation and processing*

The need for legislation to govern the levels of mycotoxins accumulating in food and animal feed was emphasised by the risks that are posed by fumonisins on human and animal health. The World Health Organizations International Agency for Research on Cancer (WHO-IARC) evaluated this risk and classified fumonisin $B_1$ as “group 2b carcinogen” (IARC, 2002). Furthermore, mycotoxin legislation could help to prevent trade barriers (Soriano and Dragacci, 2004).

Previously, mycotoxin legislation in South Africa for foodstuff included aflatoxins, *Ergot sclerotia* (Tulasne) and patulin. Recently, South Africa amended existing legislation to govern the maximum permissible levels of fumonisins and deoxynivalenol, a mycotoxin produced by *F. graminearum* (Schwabe) that causes Gibberella ear rot of maize (Government Gazette, September 2016). Restrictions were set at 4 $mg \cdot kg^{-1}$ of fumonisins ($FB_1$ and $FB_2$) for raw maize grain intended for further processing, and 2 $mg \cdot kg^{-1}$ for processed maize products for human consumption.

Currently, there is no limits set for animal feed in South Africa, however, the guidance levels set by USA food and drug administration (FDA) for fumonisins and deoxynivalenol are generally accepted (GrainSA, 2018). Enforcing these restrictions is the greatest challenge for developing countries as much of the farming is subsistence based, and does not cross legislative boundaries (Degraeve *et al*., 2015). To set up maximum allowable levels, governments need toxicological data, an understanding of how mycotoxins are distributed, consideration of legislations of other import and export countries and reliable methods of analysing mycotoxins (Van Egmond *et al*., 2007).

Urban consumers have commercial legislation to somewhat protect them, but rural consumers do not and, therefore, they have a higher exposure to fumonisin contamination leading to human and animal health issues (Rheeder *et al*., 1992). In addition to this, urban consumers and consumers in developed nations do not rely heavily on one or a few crops as a food source but have a variety of foods in their diets. Rural consumers and consumers in developing nations have a greater dependence on one or a few staple cops. This extenuates the importance of developing effective management strategies to control FER and fumonisin production.

*The role of fumonisins in disease development*

The purpose for the production of fumonisins by *F. verticillioides* is still unclear. Conflicting results have been reported for the role of fumonisins as a virulence factor (Desjardins and Plattner, 2000; Desjardins *et al.*, 2002; Proctor *et al.*, 2002; Sánchez-Rangel *et al.*, 2012). Reports have shown that non-fumonisin producing mutants are as virulent to maize as their wild type counterpart (Desjardins and Plattner, 2000; Desjardins *et al*., 2002; Proctor *et al*., 2002). Other studies mention that although fumonisins play a role in the virulence of *F. verticillioides* on maize, it is not an essential factor conferring virulence (Desjardins *et al*., 1995). On the other hand, some studies suggested that fumonisin production aids virulence by regulating pathogenesis related (PR) protein response in maize (Sánchez-Rangel *et al*., 2012), whereas further reports mention that it is an essential factor conferring pathogenicity (Glenn, *et al*., 2008).

Fumonisins is thought to provide *F. verticillioides* with a competitive advantage over other micro-organisms that share the same ecological niche (Reid *et al*., 1999). Rheeder *et al*. (1990) showed that *F. verticillioides* was able to out compete other maize rot pathogens such as *F. graminearum* (Schwabe), *Stenocarpella maydis* (Berk.) Sutton (synonym *Diplodia maydis* (Berk.) Sacc. and *F. subglutinans* (Wollenw. and Reinking). This competitive exclusion was, however, attributed more to environmental conditions. Similarly, Reid *et al*. (1999) also found *F. verticillioides* to be a highly competitive coloniser of maize, over *F. graminearum*, but again this correlation was more attributed to the environment favouring *F. verticillioides*. Reports, however, show that there exists insufficient evidence against the idea of fumonisins giving *F. verticillioides* a competitive advantage (Reid *et al*., 1999; Fox and Howlett, 2008).

Fumonisins potentially help the fungus adapt and survive within varying environmental conditions (Schmidt-Heydt *et al*., 2008). This is particularly the case for other mycotoxigenic species, such as *Aspergillus parasiticus* (Speare), *Penicillium verrucosum* (Dierckx) and *F. culmorum* (Smith) Saccardo, where abiotic factors like temperature, pH and water activity, influences the level of mycotoxins produced (Schmidt-Heydt *et al*., 2008). Likewise, abiotic factors influence the rate of fumonisin production (Picot *et al.*, 2010). *In vitro* studies have shown that water availability stress increases fumonisin production (Jurado *et al*., 2008). Additionally, fumonisin production seems to have an optimal temperature range (15-25°C) (Marín *et al*., 1999). Kim and Woloshuk (2008) stated that without the production of fumonisins, the fungus grew poorly; therefore, fumonisins could possibly have an adaptive, survival role in the growth and development of *F. verticillioides.*

Another hypothesis concerning fumonisin production is considered in response to oxidative stress (Picot *et al*., 2010). *Fusarium verticillioides* is aerobic so once the fungus infects and colonises plant tissue, the plant responds by activating an oxidative burst of reactive oxygen species (ROS) (Shetty *et al*., 2006). This deprives the fungus of oxygen and

limits further colonisation (Shetty *et al*., 2006). These ROS molecules are toxic and subsequently needs to be detoxified (Lehmann *et al*., 2015). While the rate of ROS production is greater than ROS detoxification an oxidative stress condition exists, which has been shown to stimulate fumonisin production (Reverberi *et al*., 2010). Production of mycotoxins is aimed at creating a redox state that is less harmful for fungal growth and metabolism (Reverberi *et al*., 2010). Fumonisin-producing strains of *F. verticillioides* may have an advantage by counteracting the oxidative burst by producing fumonisins (Picot *et al*., 2010; Reerberi *et al*., 2010).

## MANAGEMENT OF FUSARIUM EAR ROT AND FUMONISINS

### Cultural, chemical and biological approaches

Effective management options for FER and fumonisin contamination are limited and no registered fungicides are currently available. Biological control has been considered as a management strategy to reduce FER and fumonisin production and contamination (Alberts *et al*., 2016). Cavaglieri *et al*. (2004) looked at eleven rhizobacterial isolates and found certain *Pseudomonas solanacearum* (Smith), *Bacillus subtilis* (Ehrenberg) and *Azotobacter armeniacus* (Beijerinck) isolates to effectively reduce fumonisin ($FB_1$) production *in vitro*. The antagonistic interaction between these biocontrol agents and the pathogen is complex and not well characterised (Cavalglieri *et al*., 2004; Alberts *et al*., 2016). Biocontrol against *F. verticillioides* could eventually be used in an integrated disease management plan.

The effect of numerous cultural methods for the management of FER and fumonisins has been studied such as crop rotation, tillage, removal of weeds and other alternative hosts. Furthermore, the effect of optimal crop production, including timeous planting and harvesting, fertilisation and water supply, on FER development and fumonisin contamination has also been evaluated (Cotten and Munkvold, 1998; Munkvold, 2003b; Blandino *et al*., 2008; 2009).

Munkvold (2003b) discussed the importance of cultural practices and suggested that proper post-harvest handling and grain storage could limit FER and fumonisin accumulation, in storage. This is not the most effective method for managing the overall disease development as FER and fumonisin accumulation is strongly affected by inoculum present and environmental conditions pre-harvest (Fantazzini *et al*., 2016; Cao *et al*., 2014). The effect of maize stubble on FER was assessed by Cotton and Munkvold (1998) and found that FER pathogens are able to survive in the soil for long periods of time. They found that buried residue serves as a greater source of inoculum therefore suggested that tillage could help reduce the incidence of FER (Cotton and Munkvold, 1998). However, with the shift towards conservation agriculture (CA), more emphasis is being placed on no-till practices as a more sustainable and

environmentally sound alternative for crop production (Hobbs *et al*., 2008). Besides the lack of tillage that could affect FER incidence, CA also presents a higher risk of weed infestations, especially during the initial years of CA (Hobbs *et al*., 2008). Studies have shown that weeds could serve as alternative host crops for *F. verticillioides*, where the fungus can grow asymptomatically (Ilić *et al*., 2012; Postic *et al*., 2012). Removal of weeds is therefore an important addition to cultural management strategies. A good integrated management strategy is imperative for limiting disease development when implementing CA approaches. Further cultural practices that have been studied for their potential in FER and fumonisin management includes fertilisation and sowing time (Blandino *et al*., 2008; 2009). Blandino *et al*. (2008) assessed the effect of different nitrogen fertilisers on FER development and fumonisin accumulation and found that irrespective of the type of nitrogen source, optimal nitrogen supply could limit FER and fumonisins in maize. It was further illustrated that insufficient N application could increase the risk of FER and fumonisin accumulation and that excessive nitrogen application could increase the risk of other mycotoxins such as zearalenone (Blandino *et al*., 2008). In a study by Blandino *et al*. (2009) the role of planting time was assessed for its potential in reducing maize ear rots and mycotoxin accumulation; they found that later planting increases the risk of FER and fumonisins. They suggested that this was due to later sowing resulting in later harvest, hence maize plants spend more time in the field where conditions favour disease and mycotoxin development (Blandino *et al*., 2009).

Harvesting earlier has been associated with reduced fumonisin accumulation as the concentration was shown to increase significantly from physiological maturity until harvest (Bush *et al*., 2004). However, by harvesting earlier, grain moisture can be above 25%. This high moisture content could make both harvesting as well as post-harvest handling and storage difficult as this higher moisture content could favour fungal growth of *F. verticillioides* (as well as other opportunistic organisms) during storage and subsequent fumonisin (and other mycotoxins) contamination (Schmidt-Heydt *et al*., 2008). In South Africa, environmental conditions allow for maize to be left to dry infield as the financial impact and infrastructure to support artificial drying is not feasible. In terms of fertiliser application, reports show that increased nitrogen fertiliser could reduce mycotoxins, however, with nitrogen fertiliser being one of the greatest costs in maize production (Jones and Duncan, 1981; Reid *et al*., 2001), increasing this is neither economically or environmentally sustainable.

Different CA systems, including crop rotations and no-till practices, were tested by Mabuza *et al*. (2018) to assess the effect on FER and fumonisin accumulation in different localities in South Africa. They found that these different cropping systems had no significant effect on *F. verticillioides* or fumonisin accumulation (Mabuza *et al*., 2018). The shortfalls of cultural practices in the management of FER/fumonisins have been widely documented.

These cultural practises could contribute to the reduction of FER/fumonisins, however, their effectiveness is limited, and the implementation of these practices is not always feasible.

**Breeding for resistance to FER and fumonisin accumulation**

To date, there is no management strategy that is completely effective to prevent FER and fumonisin accumulation in maize (Horne *et al*., 2016). Plant resistance, therefore, is the most feasible management strategy as it provides an effective, sustainable approach in limiting FER/fumonisins. There are no cultivars available that offer completely immunity against FER/fumonisins. Maize inbred lines and commercial cultivars with tolerance to the disease and its mycotoxin has been reported globally (Clements *et al*., 2004; Janse van Rensburg *et al*., 2015; Rose *et al*., 2016; 2017). Conventional breeding approaches have been investigated to develop inbred lines and hybrids that have improved resistance to FER/fumonisins. A study by Hung *et al*. (2012) investigated a diallel cross with inbred lines having different levels of resistance; they suggested that the most effective resistance in hybrids exist when both parental inbred lines are resistant. Moreover, they found that this effectiveness is due to heterosis, in that the resistance observed in the hybrid was greater than the most resistant parent (Hung *et al*., 2012). It is, therefore, imperative that breeders select resistant parental material do develop resistant cultivars.

Resistance to FER and fumonisins can be found by evaluating landraces, inbred lines or hybrids. The use of resistant hybrid or inbred line germplasm reduces the lengthy process of breeding with landraces as they usually have good agronomic traits and are well adapted to local environments (Eller *et al*., 2008; Mesterhazy *et al*., 2012). Resistance to FER/fumonisins in maize is quantitatively inherited with additive gene effects being key in relaying resistance (Netshifhefhe *et al*., 2018). Recurrent selection is, therefore, a vital aspect to consider concentrating traits adding to resistance in the population (Mesterhazy *et al*., 2012). Finally, Rose (2016) investigated the use of mutation breeding to yield resistance to FER/fumonisins in maize inbred lines and identified inbred lines generating mutations that conferred improved resistance to FER/fumonisins. Conventional breeding approaches have shown improved tolerance of maize inbred lines and hybrids to FER/fumonisins; however, more knowledge is needed to understand various sources of resistance within the maize crop.

Unconventional methods of breeding can also be employed to add to resistance to *F. verticillioides* and fumonisin contamination. The genetically engineered trait of insect resistance (Bt) has been associated with reduced fungal and fumonisin contamination (Munkvold *et al*., 1999; Gatch and Munkvold, 2002; Bowers and Munkvold, 2014; Ncube *et al*., 2017). Genetically modified maize hybrids with transgenic events encoding for insecticidal proteins from *Bacillus thurigiensis* (Berliner) (Bt) indirectly influences FER severity and fumonisin accumulation (Gatch and Munkvold, 2002). Insect feeding injury causes three

interlinking problems relating to the *F. verticillioides*-maize pathosystem. Firstly, it may vector the pathogen from a diseased region to a disease-free region, whether that be from kernel to kernel, ear to ear or even plant to plant. Secondly, it produces wounds which can then serve as major entry points for the pathogen. Finally, insect feeding generally puts the crop under stress, increasing the severity of the disease and the accumulation of fumonisins (Gatch and Munkvold, 2002). Bt-technology provides a very high resistance level and targeted approach to managing insect pests of many crops including maize by reduced feed injury from certain insects thereby creating less wounds for pathogen entry (Li and Romeis, 2010). Transgenic events are specific to certain insects and there exists strong relationships between insects and disease levels. A good example of this is the relationship between *Ostrinia nubilalis* (Hubner) (European corn borer; ECB) and *F. verticillioides* (Sobek and Munkvold, 1999; Ncube *et al*., 2017). Gatch and Munkvold (2002) used different Bt-events and assessed different maize stalk rotting pathogens using the ECB. They found that *F. verticillioides* was strongly associated with ECB-injured plants, even across different Bt-events. Moreover, in a study by Clements *et al*. (2003), the Cry1Ab protein from the Mon810 transformation event was assessed and found reduced FER severity and lower fumonisin accumulation in the presence of an ECB population but not in the presence of a *Helicoverpa zea* (Boddie) (corn earworm) population. Avantaggiato *et al*. (2003) found a positive correlation between the *Sesamia nonagrioides* (Lefebvre) (pink stem borer) feeding and fumonisin accumulation in maize. Furthermore, Avantaggiato *et al*. (2003) also reported on the positive correlation between *F. verticillioides* (and *F. proliferatum*) and different stem borer insects in the family *Noctuidae*.

Enzymes with the ability to detoxify fumonisins have been identified in other species (Duvick, 2001; Varga and Tóth, 2005; Heinl *et al*., 2010). Successful transfer of these genes into maize have been accomplished, however, it is yet to be commercialised (Heinl *et al*., 2010; Alberts *et al*., 2017).

## SOURCES OF RESISTANCE

Knowledge on the various sources of resistance to FER and fumonisins within the maize plant is cardinal to a resistance breeding program. In addition, understanding how these mechanisms interact with each other and the environment, and how they relate to resistance could give further insight to breeders. The pathogen interacts with the maize plant at three intersecting levels *i.e*. at a structural, a physico-chemical and a molecular level. At each of these levels, the plant aims to defend itself against invasion by the pathogen whilst the pathogen aims to overcome each defence mechanism, so as to establish disease.

**Structural defence**

Structural barriers such as the silks, husks, and the kernel pericarp serves as the first line of defence of the maize plant that the pathogen needs to overcome. *Fusarium verticillioides* does not form penetration hyphae and, therefore, cannot breach host structural barriers (Howard, 1997). The pathogen, therefore, relies exclusively on wounds and natural openings to breech the outer barrier of the plant.

*Silks*

The maize silks are one of the main pathways the pathogen uses to colonise the maize ear (Yates and Sparks, 2008). Therefore, artificial silk inoculations are the preferred inoculation method (Munkvold and Carlton, 1997; Clements *et al*., 2003; Duncan and Howard, 2010). This potentially involves silk channel resistance factors, which is not thoroughly understood; however, silk length is thought to be a factor. Wherever the spore lands on the silk, it needs to germinate and grow down the silk and enter the kernel microenvironment before silk completely senesces (Reid and Sinha, 1998). Thus, longer, exposed silks are thought to contribute more to resistance than shorter silks as the fungus has a longer distance to grow to gain access to the kernel on longer silks (Mesterházy *et al*., 2012; Cao *et al*., 2013). Furthermore, infection via the silk channel cannot occur once the silk tissue has entirely dried out (Fig. 4) (Reid and Sinha, 1998). Reid *et al.* (2002) described the timing of senescence as a potential silk channel resistance factor and showed that susceptibility of silk tissue to *F. verticillioides* decreases as silk tissue ages. Genotypes where silk tissue dries out quicker could provide a shorter window of opportunity for fungal infection. This phenomenon could be assessed in terms of later silking genotypes as in a study by Loffler *et al*. (2010) where they found a significant correlation between decreased disease severity and later-silking genotypes. Further reports suggest that silk detachment or silk moisture content could further regulate the rate of fungal infection (Headrick *et al*., 1990). These factors, however, are largely dependent on the environment (Mesterházy *et al*., 2012). Silk channel resistance factors are not consistent across different environmental conditions and, therefore, not easily reproducible (Reid and Sinha, 1998; Mesterházy *et al*., 2012).

*Husk coverage*

Maize ears are enclosed with husk leaves that protect kernels from damage and drying out (Nickerson, 1954). Husk leaf coverage differs between varieties in terms of tightness and complete closure (Nickerson, 1954). Husks can act as a barrier for insect infestation and the subsequent fungi they may vector; and, therefore, plays a role in defence against FER

(Parsons and Munkvold, 2010). Open husks are characteristic of certain genotypes. As the ear develops it causes husk leaves to open and expose kernels (of open husked cultivars) to the environment. This is thought to increase the susceptibility of the ear to insect, bird and animal damage (Fig. 5), and subsequently increase the rate of pathogen infection (Warfield and Davis, 1996; Demissie *et al*., 2008).

*Pericarp thickness*

Once the fungus encounters the kernel, it interacts with the pericarp. This interaction is vitally important for both the fungus and the plant, for colonisation and resistance, respectively (Hoenisch and Davis, 1994). During kernel development, the structure and thickness of the pericarp varies. At about ten days after pollination, the outer wall of the of the ovary, consisting primarily of elongated parenchyma tissue, starts to modify and develop into the pericarp tissue (Kiesselbach and Walker, 1952). During this time, the pre-pericarp tissue increases in cell number and size as it becomes more compressed. This compressed tissue then becomes cellulosic consisting of dead cells as the kernel matures (Hoenisch and Davis, 1994). If the fungus overcomes host resistance mechanisms it readily colonises the pericarp tissue and produces fumonisins, before colonising the endosperm and other kernel fractions (Bryła *et al*., 2015). The kernel pericarp, therefore, has a key structural role to play in the defence against *F. verticillioides*. Sampietro *et al*. (2013) stated that a thicker pericarp is associated with a reduction in infection and lower fumonisin accumulation. Further pericarp analysis suggest that the fungus interacts with various phenylpropanoids in the pericarp and, based on the composition of these compounds within the kernel pericarp, either restricts fungal colonisation or the pathogen nonetheless gains access to the rest of the kernel microenvironment (Santiago and Malvar, 2010; Sampietro *et al*., 2013). Studies suggest that pericarp wax and other kernel factors also have a role to play in resistance to FER and fumonisin accumulation (Sampietro *et al*., 2009).

*Kernel hardness*

*Fusarium verticillioides* relies on induced wounds and other preformed openings that could be caused by insects, birds or other animals feeding on kernels to cause infection (Mesterházy *et al*., 2012). Kernel hardness (cracking ability of the kernel) refers to the pressure at which the kernel will crack open. The hardness of the kernel is predominantly determined by the hardness of the endosperm (Dorsey-Redding *et al*., 1991; Cerrudo *et al*., 2017). Harder kernels have been shown to be have less post-harvest damage (Stroshine *et al*., 1986) therefore, has been proposed to decrease the risk of fungal contamination (Magan *et al*.,

2003). Furthermore, harder kernels have correlated with lower fumonisin contamination in maize (Blandino and Reyneri, 2008).

Several methods are used to analyse maize kernel hardness including hectolitre mass (HLM), particle size index (PSI), hundred-kernel mass (HKM), protein content (PC), percentage chop (% chop), starchy and vitreous endosperm, floatation index (FI) and near infrared (NIR) spectroscopy (Almeida-Dominguez *et al*., 1997; Dombrink-Kurtzman and Knutson, 1997; Guelpa *et al*., 2015). The weight and density of kernels is used to determine the quality of the grain. Generally, grain with a higher mass generally gets a higher grade (higher quality). Kernel procedures based on weight and density include hectolitre mass (HLM), 100 kernel mass (HKM) and floatation index. Kernel HLM refers to the weight of one hectolitre of kernels, whereas HKM refers to the weight of kernels based on the weight of 100 intact kernels. Floatation index is calculated as a percentage of kernels that float in a particular solution which gives an indication of the size and of the kernels and the density of the endosperm (Almeida-Dominguez *et al*., 1997). Although mass is an important factor in terms of quality, there are other factors that play a role in the overall milling property and hardness of the kernel such as protein and moisture content, kernel fraction specifics and overall kernel density. Kernel hardness can, therefore, also be looked at in terms of differential particle size and damage susceptibility. Near infrared (NIR) spectroscopy is a method that differentiates kernel hardness based on particle size of milled kernels. According to research, NIR reflectance at a wavelength 2230 nm provides an effective differential across different genotypes, first put forward by Downey *et al*. (1986). Within this method, milled flour is sifted through a sieve of about 1 mm before being subjected to NIR spectroscopy.

Santiago and Malvar (2010) suggested that the hardness of the kernel could also be due to certain phenolic acids within the pericarp. However, susceptibility of the kernel becoming cracked could be due to several additional factors including the moisture content of the kernel, drying, handling, processing or the percentage of husk coverage. The genotype and environment have a part to play in the development of many of these factors, however, kernel hardness is still a highly heritable trait in maize (Fox and Manley, 2009). That being said, in addition to the possible resistance it can afford against *F. verticillioides*, kernel hardness is an attractive aspect to select for growers and breeders.

**Physico-chemical properties**

Following successful access to the kernel microenvironment, the pathogen interacts with the maize plant at a physico-chemical level where the pathogen again relies on its ability to proliferate and grow in this environment. Changes within the kernel microenvironment, as kernels mature, are thought to play a major role in the regulation of FER and fumonisin production by *F. verticillioides* (Picot *et al*., 2010; 2011; Smith *et al*., 2012).

*pH*

Kernel pH changes as the kernel develops and influences the accumulation of fumonisins (Picot *et al.*, 2011; Smith *et al*., 2012). According to Smith *et al*. (2012) acidic conditions (pH 5) within the kernel microenvironment are more conducive and alkaline conditions (pH 8) which are repressive for fumonisin production. This interaction is further complicated by whether the kernel pH itself influences the rate of fumonisin production or whether the fungus manipulates the kernel microenvironment to induce favourable conditions for fumonisin production (Marin *et al*., 2004). The dent stage (R5) of kernel development is considered the most conducive for fumonisin production due to the combination of a low pH and high levels of amylopectin (Picot *et al*., 2011). High levels of starch in the dent stage provides the pathogen with a glucose supply that causes acidification when catabolised by the pathogen (Brandao *et al*., 1992). Kim and Woloshuk (2008) further showed that when they amended blister stage (R2) kernels (which have low amylopectin content) with amylopectin, they found that it lowered kernel pH to acidic levels, making it more conducive for fumonisin production. Bluhm and Woloshuk (2005), further, noted that dent stage kernels had a dramatically increased rate of fumonisin accumulation compared to blister and milk stage kernels, prior to a significant decrease in the pH. Picot *et al*. (2011) also reported a rapid accumulation of amylopectin during the first stages of kernel development and that fumonisin biosynthesis only appeared three weeks later. Therefore, although acidic conditions are conducive for fumonisin accumulation, whether it be a prerequisite or result of fumonisin production is not known.

*Moisture content*

Moisture content has been identified as a factor that regulates fumonisin biosynthesis by *F. verticillioides* (Jurado *et al*., 2008; Schmidt-Heydt *et al*., 2008). Kernel moisture content is thought to provide water for fungal activity, such as growth and metabolism (Schmidt-Heydt *et al*., 2008). This is particularly important during the early stages of kernel development when the higher moisture content may create a more conducive environment for fungal growth (Jurado *et al*., 2008; Picot *et al*., 2011). However, *F. verticillioides* has been described as a xerophilic fungus in that it is able to grow at lower water availabilities than other *Fusarium* species (Marín *et al*., 2004). Water stress within the kernel microenvironment has been shown to increase the expression of *FUM1* gene, a key regulator of fumonisin biosynthesis (Jurado *et al*., 2008). As the maize kernel develops, starch accumulates, and the moisture content decreases causing water availability stress. The strain on the fungus activates *FUM1* gene expression initiating fumonisin biosynthesis (Jurado *et al*., 2008; Schmidt-Heydt *et al*., 2008). Water availability stress is further increased under drought conditions and as a consequence,

increases fumonisin production (Mutiga *et al*., 2015). Furthermore, the decrease in water availability during the post-physiological maturity stage, while kernels are drying, correlates with an increase in fumonisin accumulation (Picot *et al*., 2011).

*Sugar and starch*

Early stages of kernel development have a high sugar (glucose) content which is converted to starch molecules in more mature kernels (Bluhm and Woloshuk, 2005). *Fusarium verticillioides* ultimately hydrolyses starch and sugar molecules to utilise as a carbon source for growth and metabolism (Kim *et al*., 2011). Two major starch polymers are found in maize kernels namely amylose and amylopectin and Bluhm and Woloshuk (2005) noted imperative differences in *in vitro* $FB_1$ production rates between the two starch molecules. When amylopectin was the source of starch, *F. verticillioides* cultures produced considerably more $FB_1$ than cultures grown on amylose or even sugar monomers such as glucose or dextrin (Bluhm and Woloshuk, 2005). And, *in planta*, a significant positive relationship has been shown between sugar content and fungal biomass (Van Zyl, 2015). Sugar molecules, like glucose, causes an activation of the H+- ATPase proton pump in the membrane of the fungus resulting in extracellular acidification as shown by Brandao *et al*. (1992) when looking at *Fusarium oxysporum* (Schlecht. Emend. Snyder and Hansen) species. This kind of acidification could be part of the reason for the increase in fumonisin production when certain starch and sugar molecules are present. However, is not quite clear whether acidification is the result of starch hydrolysis or it is a pre-requisite for fumonisin production.

*Nitrogen and carbon*

Nitrogen is an important factor for growth and development of fungal pathogens (Fox and Howlett, 2008). Nitrogen sources are able to act as activators of global regulatory genes (Kim and Woloshuk, 2008). AREA is a gene that encodes for a major nitrogen regulatory protein required for nitrogen catabolism and is also required for fumonisin production (Kim and Woloshuk, 2008). When preferred nitrogen sources become limiting within the fungal environment certain genes are expressed that allows the pathogen to utilise other sources of nitrogen. Activation of these genes requires global regulators, such as AREA and NIT2 (Caddick *et al*., 1986; Kim and Woloshuk, 2008). This could explain why there is an increase in fumonisin production when the pathogen is under stress. More important than specific nitrogen sources, is the ratio of carbon to nitrogen (C/N). Blister stage kernels accumulate low levels of fumonisins possibly due to the low C/N in the kernel microenvironment (Kim and Woloshuk, 2008). The C/N ratio not only regulates fumonisin biosynthesis, but also has an effect on the increase in fungal biomass (Picot *et al*., 2010). Jimenez *et al*. (2003) showed that

an increase in the C/N ratio increased the production of fumonisins and also decreased the rate of fungal growth in liquid cultures. Blister stage kernels has a high level of free amino acids and very limited starch; therefore, it is not conducive for fumonisin production as opposed to mature kernels, with a high level of starch and proteins, creating a favourable C/N environment for fumonisin production (Bluhm and Woloshuk, 2005; Kim and Woloshuk, 2008). When nitrogen sources are limited the C/N ratio is decreased and this could once again elude to the reason for the increase in fumonisin biosynthesis and decrease in fungal biomass accumulation under such conditions.

*Phenolic acids*

Phenolic compounds are naturally occurring secondary metabolites produced by plants (Shaw *et al*., 1990; Kähkönen *et al*., 1999). They are synthesised through the shikimate pathway where carbohydrates (primary metabolites) undergo metabolism to produce aromatic amino acids (Herrmann and Weaver, 1999). Two specific aromatic amino acids, tyrosine and phenylalanine, produce 4-coumaric acid and cinnamic acid through the phenylalanine ammonia lyase (PAL) pathway (Shaw *et al*., 1990). These compounds are further converted to an array of known phenolic compounds through the phenylpropanoid pathway (Tarnawski *et al*., 2006).

Phenolic acids are the most abundant form of phenolic compounds in maize grain, with ferulic acid being the predominant phenolic acid, followed by ρ-coumaric acid (Zhang *et al*., 2017). Phenolics can be free (soluble) or bound (insoluble) to certain components of the cell wall (Salinas-Moreno *et al*., 2017). In maize grain free phenolic acids provide a more diverse array of compounds than bound forms (Pozo-Insfran *et al*., 2006; Cuevas-Montilla *et al*., 2011). Most free phenolic acids are concentrated in the germ and, to a lesser extent, the endosperm (Das and Singh, 2016). Free phenolic acids, however, are present in very low amounts when compared to bound phenolic acids. Bound phenolic acids are mostly bound to polysaccharides, such as hemicellulose and lignans in the cell wall (Wallace and Fry, 1995). In maize kernels, phenolic compounds are mainly situated in the outer layers of the grain such as the aleurone layer and the pericarp tissue (Sampietro *et al*., 2013; Das and Singh, 2015). The role of phenolic compounds in defence has become a topic of focus in recent years. Phenolic acids are not only present constitutively but can be induced in response to external biotic and abiotic stress factors (Dixon and Paiva, 1995). Phenolic compounds have been illustrated to be antifungal against *F. verticillioides* growth and reduce fumonisins *in vitro* (Beerkrum *et al*., 2003; Sampietro *et al*., 2013; Salinas-Moreno *et al*., 2017). Therefore, recent studies have focussed on understanding the mechanisms of phenolic compounds to determine how it may be employed for the management of mycotoxigenic fungi and/or mycotoxin accumulation (Boutigny *et al*., 2008; Picot *et al*., 2013). Phenolic compounds can

form part of chemical or physical barriers (Dixon *et al*., 2002). When the fungus infects, phenolic acids have been shown to be quickly accumulate at the site of infection (Picot *et al*., 2010). This response could be due to phenolic compounds being released from the cell wall as well as additional biosynthesis of certain phenolic compounds (Picot *et al*., 2013). Induced phenolics may be used to reinforce the cell walls of healthy plant cells, inactivate key fungal enzymes or be broken down to compounds like quinones that are also toxic to fungi (Kim *et al*., 2004; Boutigny *et al*., 2008; Ponts *et al*., 2011). Phenolic compounds are thought to inhibit fungal growth by disrupting the integrity of fungal cell membranes (Passone *et al*., 2005; Ponts *et al*., 2011). Phenolic compounds limit the production of fumonisin biosynthesis in *F. verticillioides* possibly by disrupting the expression of fumonisin producing genes (Beekrum *et al*., 2003) or directly detoxify accumulated fumonisins and other mycotoxins (Boutigny *et al*., 2012). Das and Singh (2016) suggested that the detoxification by phenolic compounds is due to its ability to form stable bonds with free radical molecules by donating a proton from its hydroxyl group. Furthermore, the potential of phenolic acids to be used as a post-harvest application to reduce fumonisin accumulation has been discussed (Ferrochio *et al*., 2013) and could provide another aspect of management of *F. verticillioides* and its associated mycotoxins.

**Molecular defence to *Fusarium verticillioides***

Resistance to FER in maize has been shown to be polygenic with moderate to high heritability within breeding lines (Pérez-Brito *et al*., 2001; Robertson-Hoyt *et al*., 2006; Rose *et al*., 2016). Differential gene expression of certain genes, in response to *F. verticillioides*, has been demonstrated which predisposes the plant to either be resistant or susceptible (Lanubile *et al.*, 2010; 2014). Some of these genes could be manipulated by the pathogen to create a more conducive environment for the pathogen's growth, metabolism and fumonisin production, whereas other host genes could be expressed to inhibit further colonisation of the pathogen within the crop.

Plants have developed a two-layered defence mechanism that is differentiated based on signalling pathways, that being pattern recognition receptor (PRR) and resistance gene (*R*-gene) receptor mechanisms (Aderem and Ulevitch, 2000). Within the first layer, PRR's recognise conserved pathogen characteristics called pathogen associated molecular patterns (PAMPs) which initiates a PAMP-triggered immunity (PTI) (Aderem and Ulevitch, 2000; Jones and Dangl, 2006; Koeck *et al.*, 2011). As *F. verticillioides* colonises maize, it produces chitin and β-1,3-glucan which are detected by PRRs that activate downstream signalling (Sánchez-Rangel *et al*., 2012). This induces a PTI by producing hydrolytic enzymes such as β-1,3-glucanases and chitinases which are involved in degradation of fungal cell walls (Sánchez-Rangel *et al*., 2012). These hydrolytic enzymes are pathogenesis related (PR) proteins that

have shown to have antifungal properties *in vitro*, with chitinases part of PR3 and β-1,3-glucanases part of PR2 family of proteins (Sánchez-Rangel *et al*., 2012; Sinha *et al*., 2014). Wang *et al*. (2016) suggested that within the *F. verticillioides*-maize pathosystem, PTI plays a crucial role in defence.

Within the second layer, resistance (R) gene receptor mechanisms are initiated by the recognition of pathogen effector molecules and results in an effector triggered immunity (ETI) (Horbach *et al*., 2011). Effector molecules are, in essence, the response of the pathogen to PTI and function to overcome this immunity. The R-gene proteins detect very specific effector molecules (Koeck *et al*., 2011). This second layer, generally, provide a more specialised defence mechanism providing a higher level of resistance than a PTI (Koeck *et al*., 2011). Within the *F. verticillioides*-maize pathosystem a number of R-proteins have been described including proteins within the coiled coil-nucleotide binding site-leucine rich receptor (CC-NBS-LRR) and NBS-LRR families (Wang *et al*., 2016; Shu *et al*., 2017). Effector molecules are not restricted to proteins but can also be secondary metabolites. Within the *F. verticillioides*-maize pathosystem fumonisins play a vital role as effector molecules during maize colonisation (Arias *et al*., 2012).

In parallel with pathogen recognition, signalling molecules such as $Ca^{2+}$ are probably due to calcium dependent protein kinases (CDPK) (Lehmann *et al*., 2015). The burst of $Ca^{2+}$ activates respiratory burst oxidase homolog (RBOH) protein (Lanubile *et al*., 2010) that results in a burst of reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$), superoxide ($O^{2-}$), hydroxyl radical ($OH^-$) or hydroperoxyl radical ($HO^{2-}$) (Shetty *et al*., 2007; Lehmann *et al*., 2012). This burst of ROS causes programmed cell death (PCD) (Lehmann *et al*., 2015) and is aimed at isolating the pathogen by reinforcing structural barriers to limit its spread. Within the *F. verticillioides*-maize pathosystem the generation ROS are strongly associated with $Ca^{2+}$ signalling that can result in hypersensitive responses and PCD (Sánchez-Rangel *et al*., 2012; Wang *et al*., 2016; Shu *et al*., 2017). The plant then also produces scavenger molecules such as peroxidase to protect itself against oxidative stress (Lanubile et al., 2010; Sánchez-Rangel *et al*., 2012). These mechanisms are associated with a number of genes including PR-proteins, detoxification enzymes or β-glucosidases (Lanubile *et al*., 2010). Whether or not specific defence related genes are as stable and how much they correlate with different structural and physico-chemical factors within commercial cultivars, as they do in breeding lines, is not well defined.

Pathogenesis-related (*PR*) genes are common in most plants and forms part of a complex defence mechanism. PR proteins consist of about 17 known families of proteins (Eulgem, 2005; Sinha *et al*., 2014). *PR* gene expression has been showed to be associated with a number of initial defence mechanisms such as PCD, cell wall modifications and production of anti-microbial proteins (Eulgem, 2005). They are aimed at protecting the plant against different

kinds of stresses such as pathogen attack, physical or chemical wounding and adverse growing conditions (Sinha *et al*., 2014). PR proteins are able to endure harsh microenvironments such as very acid conditions where many other proteins may be denatured (Stintzi *et al*., 1993). Furthermore, they are very tolerant to proteolytic enzymes in the plant (Stintzi *et al*., 1993; Sinha *et al*., 2014). Certain families of PR genes are antimicrobial such as *PR1*, *PR4* and *PR5*. Some of which have distinctly different expression profiles between maize inbred lines resistant and susceptible to *F. verticillioides*. A study by Lanubile *et al*. (2010) showed *PR1*, *PR5* and *PRm6*, amongst others, were already being expressed prior to inoculation with *F. verticillioides* in resistant maize lines as conversely in the susceptible lines, these genes were greatly synthesised, from a basal level, only after inoculation. *PR1* seems to be an eminent family of PR proteins. It was first reported in 1970 within the tobacco-TMV pathosystem (Van Loon and Van Kammen, 1970). Beyond this, it was shown to consist of some of the most abundantly produced family of PR proteins during pathogen attack (Breen *et al*., 2017). However important, these proteins do not work alone, and defence is achieved by a combination of different proteins and mechanisms all working together to limit pathogen invasion and disease progression within the plant.

## CONCLUSION

Maize stands as the most dominant food crop and serves as a staple crop for both urban and rural consumers. It is also an export commodity thereby earning South Africa foreign income. FER of maize, caused by the mycotoxigenic fungi, *F. verticillioides* is a serious disease, causing reduced yield and quality of maize. The disease and the subsequent contamination of grain with mycotoxins, therefore, pose a major threat to both food security and food safety. Resistant cultivars are the most feasible method for controlling the disease although a number of factors influence resistance to FER and fumonisin contamination. The plant has, therefore, developed several mechanisms that could aid in its defence against the pathogen including structural barriers, physico-chemical properties and resistance genes.

Therefore, the aim of **Chapter 2** was to compare phenotypic, physico-chemical and genetic characteristics of maize inbred lines and cultivars resistant or susceptible to FER and fumonisin accumulation. By juxtaposing these characteristics, the purpose was to determine any correlation between different levels of resistance (structural, biochemical or genetic) and/or characteristics that potentially contribute to resistance to *F. verticillioides* and fumonisins.

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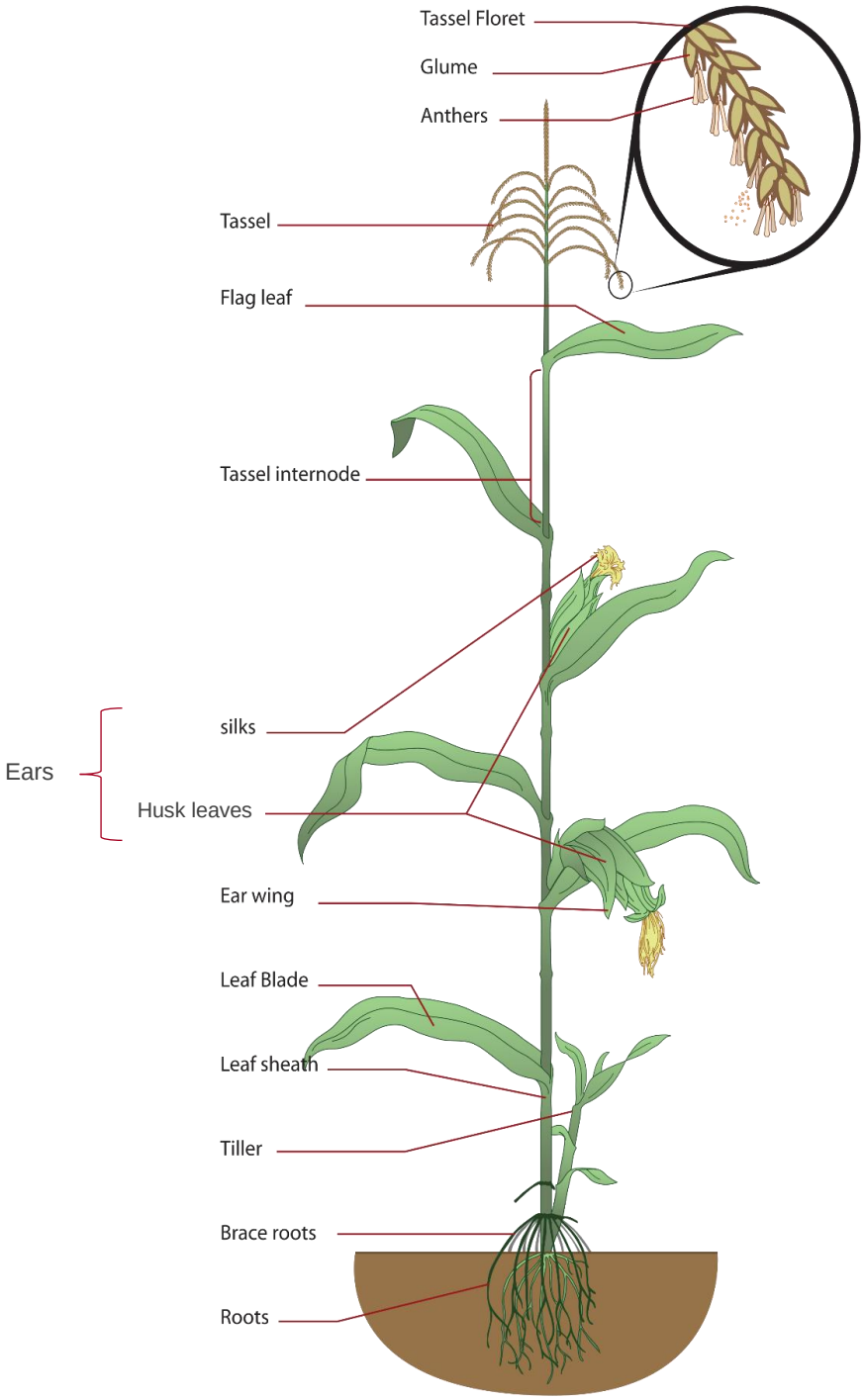


**Figure 1**. Physical characteristics of the maize plant.

(Source: https://upload.wikimedia.org/wikipedia/commons/9/98/Maize_plant_diagram.svg)

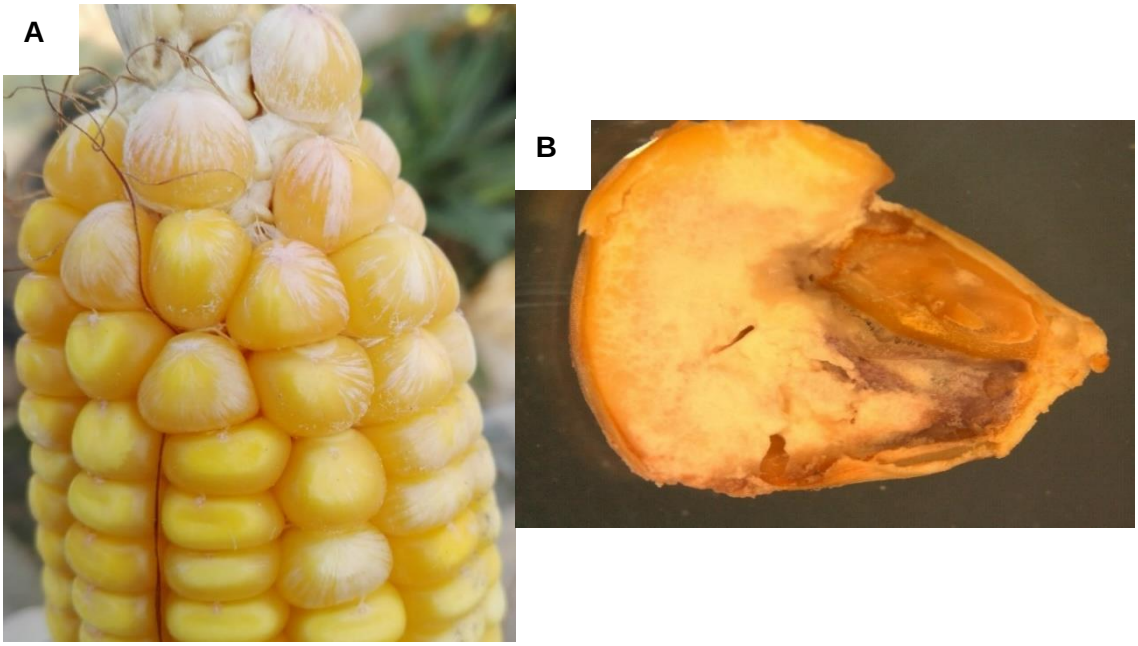


**Figure 2**. Symptoms of Fusarium ear rot of maize caused by *Fusarium verticillioides*. A: starburst symptoms, B: degradation of kernel tissue.

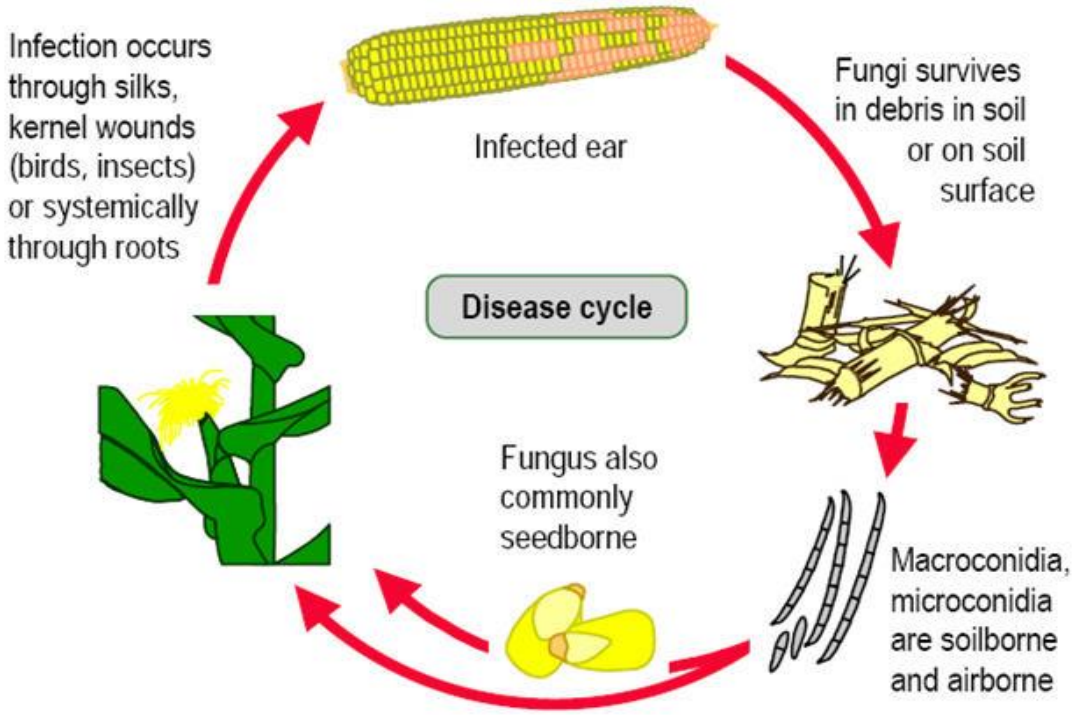


**Figure 3**. The disease cycle of Fusarium ear rot of maize caused by the fungal pathogen *Fusarium verticillioides*.
https://www.pioneer.com/home/site/us/agronomy/crop-management/corn-insect-disease/fusarium-ear-rot/

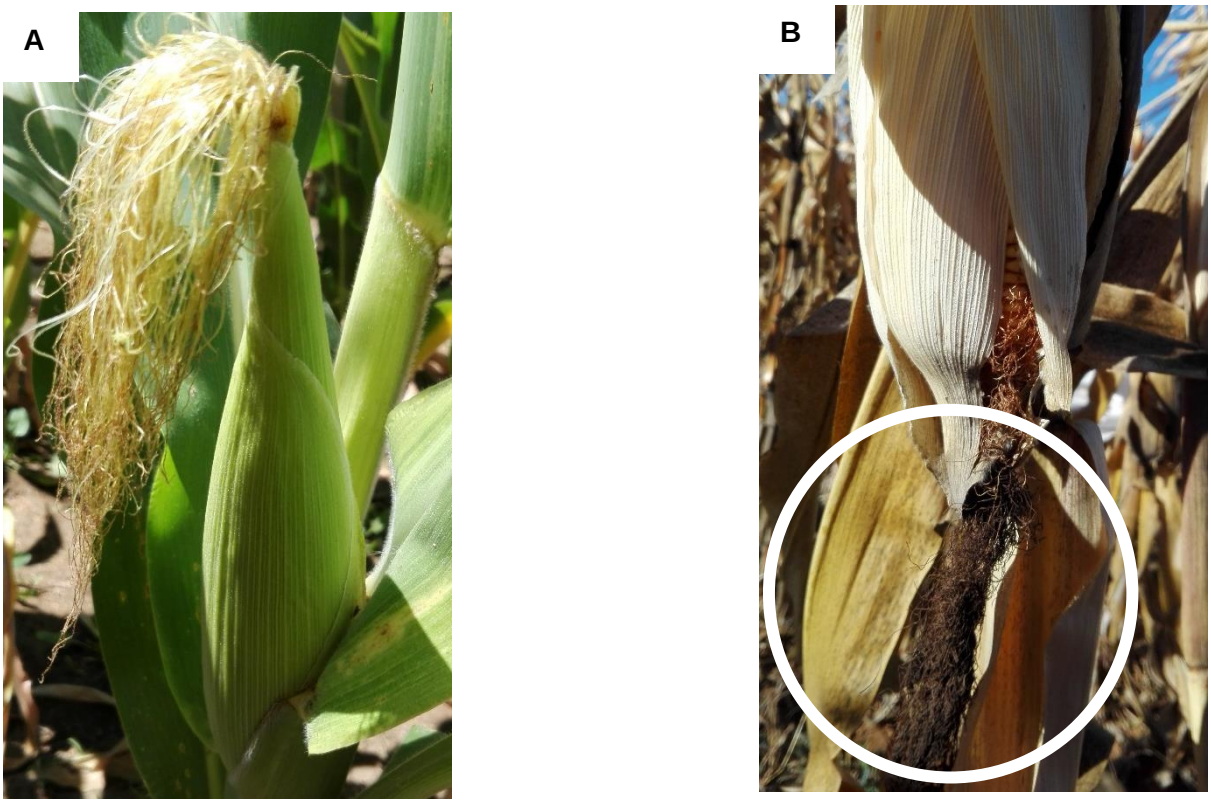


**Figure 4.** Maize ear with exposed silk tissue. A: silk tissue that is receptive to active pollen at anthesis. B: silk tissue that has been completely dried out (senesced) at harvest.

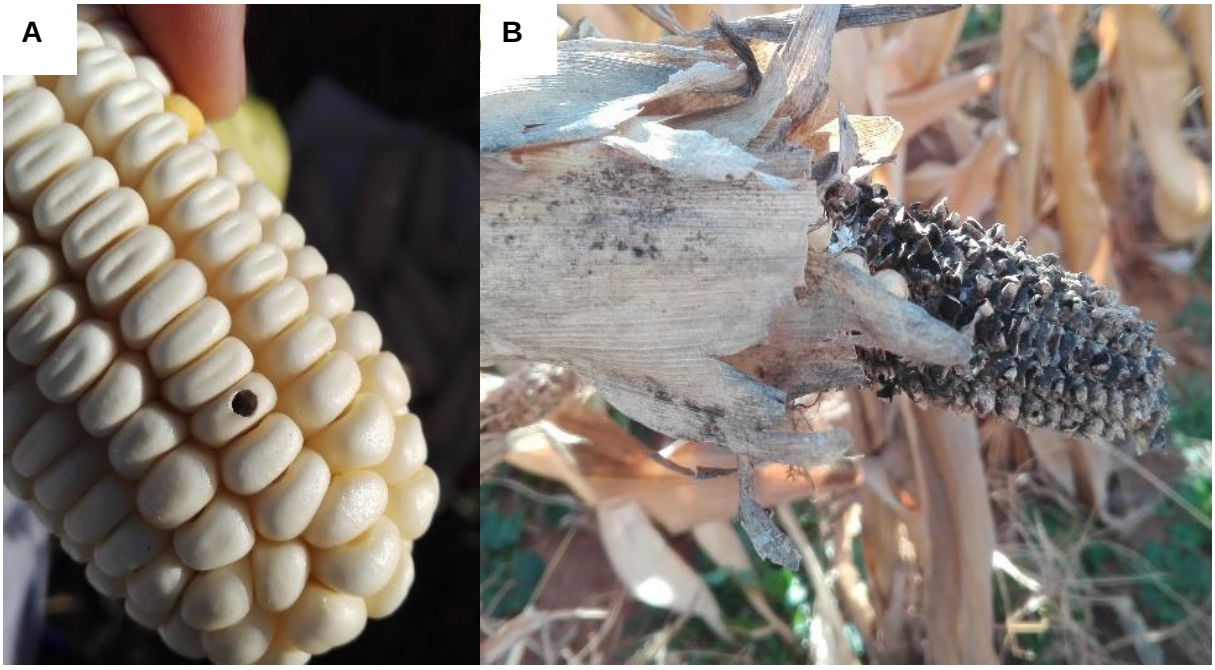


**Figure 5**. Maize ears damaged by (A) insects and (B) birds or animals when grown in the field.

# CHAPTER 2

## Structural, physico-chemical and genetic traits of maize associated with resistance to *Fusarium verticillioides*


### ABSTRACT

Fusarium ear rot (FER) of maize, primarily caused by the fungal pathogen *Fusarium verticillioides*, can reduce grain yield and grain quality. The fungus causes kernel rot and produces a range of mycotoxins, of which fumonisins are by far the most abundantly produced. Fumonisins have been associated with several health implications in humans and animals. Management options for the disease are limited as there are no registered fungicides available and the effect of cultural practices and biological control options are often inadequate. Plant resistance presents the most effective and sustainable approach to manage FER/fumonisin accumulation in maize. Resistance to *F. verticillioides* may be governed by numerous factors, including phenotypic, physico-chemical and genetic properties of the maize plant. In this study, the structural, biochemical and genetic basis of resistance to *F. verticillioides* was investigated in a greenhouse trial and two field trials. These trials contained previously characterised inbred lines as well as uncharacterised commercial cultivars. The phenotypic data collected comprised silk length, husk coverage, pericarp thickness hundred-kernel mass and kernel hardness; the physico-chemical data collected consisted of kernel pH, moisture content, total nitrogen and carbon as well as phenolic acids, and the genetic data evaluted pathogenesis-related genes (*PR1* and *PR5*) and as well as *peroxidase* gene expression. This data was then correlated to FER severity, fumonisin accumulation and fungal DNA (infection indicators). Disease development and fumonisin contamination in Vaalharts was significantly more than in Potchefstroom. Kernel pH was the most important trait associated with disease development and was negatively correlated to all infection indicators. The carbon:nitrogen ratio was also negatively correlated to infection indicators in the cultivar trial. *Peroxidase* and *PR5* gene expression had significant positive correlations with infection indicators in both trials and the *PR1* gene in the inbred line trial. The most evident gene expression was found in susceptible genotypes. There were no strong correlations between phenotypic characteristics and infection indicators. The results in this study could aid breeders and growers in selecting material resistant to FER/fumonisin accumulation more effectively.

## INTRODUCTION

Fusarium ear rot (FER) of maize is caused by *Fusarium verticillioides* (Saccardo) Nirenberg, *F. proliferatum* (Matsushima) Nirenberg, *F. subglutinans* (Saccardo) Nirenberg and *F. temperatum* (Scauflaire and Munaut) (Schoeman *et al*., 2018). These are all mycotoxigenic pathogens that affect maize (*Zea mays* L.) at all stages of development (Foley, 1962; Gelderblom *et al.*, 1988; Munkvold, 2003a). Of the FER pathogens, *F. verticillioides* is considered most important in Southern Africa (Boutigny *et al*., 2012; Schoeman *et al*., 2018).

The contamination of maize grain with *F. verticillioides* is a major constraint to maize production. It causes a reduction in yield and quality to commercial and subsistence farmers around the world (Presello *et al*., 2008; Geber *et al*., 2010). *Fusarium verticillioides* causes the rotting of maize kernels and produces a range of toxic secondary metabolites, called mycotoxins, of which fumonisins are the most important (Gelderblom *et al.*, 1988). Fumonisin-contaminated maize cause health implications in humans and animals including oesophageal cancer in humans, neural tube defects in new-born babies, leukoencephalomalacia in horses as well as liver and kidney damage in sheep (Kriek et al., 1981; Marasas et al., 1981; 1984; 1988; 2004).

The success of the pathogen is largely attributed to the hemi-biotrophic nature that allows it to colonise plant tissue endophytically or later become a necrotroph (Bacon and Hinton, 1994; Bacon *et al*., 2008). The silking period (anthesis) is a crucial stage for infection of maize with *F. verticillioides* (Munkvold and Carlton, 1997; Clements *et al.*, 2003). At this stage the kernel microenvironment favours the endophytic development of the pathogen, allowing it to proliferate without necessarily causing cell death (Wicklow *et al*., 2005). Induction of the necrotrophic phase is related to biotic and abiotic stress endured by the pathogen (Bacon and Hinton, 1994; Bacon *et al*., 2008). These stress factors can include other microorganisms competing for nutrients, plant cell pH, water availability, plant drought or water logging (Bacon *et al*., 2001; 2008). Although fumonisins have been shown to accumulate during the endophytic phase, it is strongly associated with the necrotrophic phase (Bacon and Hinton, 1994; Desjardins *et al*., 1995; Bacon *et al*., 2008). Furthermore, the disease is also strongly driven by the environment (Pérez-Brito *et al.*, 2001; Robertson-Hoyt *et al.*, 2006; Rose *et al.*, 2016). Warmer, dryer conditions, especially around silking, increase the rate of successful infection and therefore increasing the likelihood of disease development and fumonisin contamination (Cao *et al*., 2014).

Management of FER/fumonisins is particularly challenging (Munkvold, 2003a). Successful practices for managing the disease are limited. Cultural practices are aimed at creating an environment that is less conducive for infection and disease development in the field (Munkvold, 2003b). Practices such as fertilisation methods, biocontrol agents, application

of insecticides, tillage, crop rotation and adjusting planting and harvesting dates have been investigated (Flett *et al.*, 1998; Munkvold, 2003b; Pereira *et al.*, 2007; Alakonya *et al.*, 2008; Parsons and Munkvold, 2010; Mabuza *et al*., 2018). Cultural practices are by and large ineffective in controlling *F. verticillioides* in the field especially when environmental conditions are conducive for disease and mycotoxin development (Munkvold, 2003c; Cao *et al*., 2014). Plant resistance presents a feasible method to control FER and fumonisin accumulation (Munkvold, 2003b; Maschietto *et al*., 2017). However, breeding for resistance to *F. verticillioides* is challenging as both the genetics of the host and the pathogen need to be considered (Aquaah, 2007). The strong environmental effect and polygenic nature of resistance to *F. verticillioides* further complicate breeding endeavours (Pérez-Brito *et al.*, 2001; Robertson-Hoyt *et al.*, 2006; Lanubile *et al.*, 2017). A number of factors contribute to FER/fumonisin-resistance within maize inbred lines such as phenotypic, physico-chemical and genetic factors (Picot *et al*., 2010; Mesterhazy *et al*., 2012). These may include silk length and husk coverage, kernel hardness phenolic compounds, kernel pH and genes for plant defence. However, the relative importance of all these factors and how they correlate to each other are not well characterised.

Resistance in maize to *F. verticillioides* is mediated at different levels. Initially successful infection of maize kernels depends on the fungus crossing certain barriers. These include the length of maize silks, duration of silking, silk wetness and husk coverage (Warfield and Davis, 1996; Reid *et al.*, 1992; 2002; Stewart *et al.*, 2002; Maiorano *et al.*, 2009; Parsons and Munkvold, 2010). Susceptibility of maize has been documented to increase with shorter silks, faster silk senescence and incomplete husk coverage. Yet, a resistant genotype with open husks is less prone to FER than a susceptible genotype with open husks (Warfield and Davis, 1996). The maize kernel has certain structural features that can contribute to resistance *i.e*. the pericarp layer. The pericarp layer is the outermost layer, providing the kernel with defence against the external environment (Kiesselbach and Walker, 1952). Studies have shown that maize kernels with a thicker pericarp had lower fumonisin levels than ones with thinner pericarps (Sampietro *et al*., 2013). Additionally, the pericarp has different layers contributing to the overall hardness of the kernel (Fox and Manley, 2009). Furthermore, hardness can also be indicated by several other kernel factors such as the vitreous and starchy endosperm, size, weight and density (Guelpa *et al*., 2015). Tolerance to cracking of kernels indirectly contributes to resistance to pathogens by preventing wounds that can act as infection sites for *F. verticillioides* (Sampietro *et al.,* 2013). Moreover, kernel hardness also protects kernels against insect feeding that provide infection pathways for *F. verticillioides* (Schulthess *et al*., 2002; Lale and Kartay, 2006; Abebe *et al*., 2009; Mwololo *et al*., 2013; Zunino *et al*., 2015).

Following successful breech of structural barriers, *F. verticillioides* interacts with the kernel microenvironment where resistance to the fungus may be mediated at a physico-chemical

level (Picot *et al.*, 2010). Since anthesis is a crucial period for fungal infection, understanding the physico-chemical interaction at this point could aid in the understanding of resistance to *F. verticillioides*. During this period, developing kernels has an alkaline pH and high moisture content (Warfield and Gilchrist, 1999). Reports suggest that the fungus has mechanisms in place, such as the PAC1 protein, to allow fungal growth under these conditions (Flaherty *et al*., 2003). Fumonisin production, however, is reportedly suppressed at alkaline conditions (Shim *et al*., 2003). As kernel development progresses, nitrogen and free water becomes more limiting and kernel pH becomes more acidic (pH 3.0-4.0) (Keller *et al*., 1997). This is correlated with an increase in fumonisin production (Keller *et al*., 1997; Shim *et al*., 2003; Jurado *et al*., 2008). Furthermore, maize kernels inoculated with *F. verticillioides* has been shown to have a lower pH than water inoculated kernels (Van Zyl, 2015). Therefore, maize genotypes that are predisposed to factors such as higher kernel pH or nitrogen content during kernel development could also indirectly contribute to resistance to FER (Marin *et al*., 2004). Phenolic compounds in maize kernels may play an active role in resistance to fungal pathogens (Zabka and Pavela, 2013; Salinas-Moreno *et al*., 2017). Phenolic compounds are secondary metabolites produced by plants both constitutively and in response to external stimuli (Ponts *et al*., 2011). They limit the growth of *F. verticillioides* as well as break down fungal cell membranes (Boutigny *et al*., 2008; Ponts *et al*., 2011). Furthermore, they have been shown to detoxify fumonisins *in vitro* (Beerkrum *et al*, 2003; Ferrochio *et al*., 2013). The potential of these compounds is immense as they could provide an environmentally-sound source of resistance to add to the arsenal of defence mechanisms available in the maize plant.

The molecular basis of resistance to FER and fumonisin accumulation has been extensively investigated in maize (Lanubile *et al.*, 2010; 2014; Maschietto *et al.*, 2016; Lanubile *et al.*, 2017). This includes the induction of pathogenesis-related (*PR*) genes following pathogen infection (Breen *et al*., 2017). PR proteins are classified into 17 families, of which most have antifungal activity while others have antiviral and antibacterial activity (Sinha *et al*., 2014; Breen *et al*., 2017). They are effective at low concentrations. *PR* genes are expressed in all plant-pathogen interactions in an attempt to inhibit pathogen colonisation (Sinha *et al*., 2014; Maschietto *et al*., 2016). Lanubile *et al*. (2010) reported that *PR1*, *PR5, PRm3* and *PRm6* were found in a maize inbred line susceptible to *F. verticillioides* when compared to a tolerant line prior to inoculation, but that their expression was increased 48 hours after inoculation (Lanubile *et al.*, 2010). The PR5 protein breaks down the integrity of fungal cell membranes, thereby inhibiting fungal growth (Sinha *et al*., 2014). Genes that protect maize kernels from oxidative stress *i.e*. peroxidase have also been associated with resistance in maize to *F. verticillioides* (Maschietto *et al.*, 2016; Lanubile *et al*., 2017).

After infection, the plant produces reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) and super oxide ($O_2^-$) (Shetty *et al*., 2006). ROS can have an antimicrobial

effect against pathogens that results in hypersensitive cell death (Alvarez *et al*., 1998) and play an important role in secondary signalling (Leon *et al*., 2001). During the pathogen's necrotrophic lifecycle, ROS molecules are detoxified by peroxidases, catalases and superoxide dismutases (Shetty *et al*., 2006; Lehmann *et al.*, 2015).

There has been much progress in terms of understanding the maize-*F. verticillioides* pathosystem and how individual characteristics relate to resistance in maize inbred lines. However, there is limited understanding of these characteristics relate to each other or how the collective knowledge can be applied to identify resistant genotypes. The purpose of this study was to investigate host resistance traits at the structural, physico-chemical and genetic level in well-characterised inbred lines (glasshouse trial) and commercial maize cultivars (field trials) resistant or susceptible to FER and fumonisin accumulation. The role of these traits in plant defence will also be determined. Information obtained in this study would help breeders and producers to select FER/fumonisin-resistant material.

## MATERIALS AND METHODS

### Plant Material

*Cultivar and inbred line trials*

Four well-characterised maize inbred lines were used for the greenhouse study. These include two lines resistant to FER and fumonisins (CML 390 and CML 444), one line susceptible to FER and fumonisins (R2565y), and one line susceptible to FER but resistant to fumonisins (CB 222) (Table 1). The trial was conducted under greenhouse conditions at the Welgevallen research farm at Stellenbosch University, South Africa. Plants were grown in 15-L planting bags in a mixture of potting soil and sand (1:1). The trial was irrigated and fertilised twice a day using drip irrigation with nutrition and irrigation provided as required. The trial was planted in a randomised complete block design with three biological replicates per treatment. Pollination was performed manually by collecting pollen in paper bags and depositing it on receptive stigmas. After physiological maturity, plants were allowed to dry for 1 week by turning off the irrigation before the primary ears were harvested.

Fifteen commercial cultivars, planted in the national cultivar trial conducted by the Agricultural Research Council's Grain Crop Institute during the 2016/17 season, were randomly selected used in this study (Table 2). The cultivars were planted in two localities; Potchefstroom (grid ref.: 26730 S, 27070 E; altitude, 1349 m) and Vaalharts (grid ref.: 27950 S, 24830 E; altitude, 1180 m) representing dryland and irrigation production, respectively. Each cultivar was planted in three field plots in a randomised complete block design at each locality. A field plot consisted of two 10-m rows with 1 m spacing between rows. Each row

consisted of 25-30 plants. Maize plants were allowed to dry naturally infield for 4 to 6 weeks before being manually harvested per row.

**Artificial inoculations**

The field and greenhouse trials were artificially inoculated at anthesis with the highly pathogenic and virulent *F. verticillioides* isolate MRC 826 (Programme on Mycotoxins and Experimental Carcinogenesis, Medical Research Council (PROMEC-MRC), Tygerberg, South Africa). Fungal inoculum was prepared by growing *F. verticillioides* MRC 826 on potato dextrose agar (PDA) for about 5 to 7 days at 25ºC. Hyphae were subsequently transferred to 100 mL of Armstrong media to promote sporulation. Armstrong media was prepared according to Booth (1971) as follows: 0.02 mg $L^{-1}$ sucrose, 0.0004 g $L^{-1}$ magnesium sulphate, 0.0016 g $L^{-1}$ potassium chloride, 0.0011 g $L^{-1}$ potassium dihydrogen phosphate, 0.0059 g $L^{-1}$ calcium nitrate, 20 µL $L^{-1}$ ferric chloride, 20 µL $L^{-1}$ manganese sulphate, 20 µL $L^{-1}$ zinc sulphate suspended in sterile deionised $H_2O$ before being autoclaved. Spores were produced in 100 mL of Armstrong medium and incubated at 25ºC for 4 to 5 days while agitated at 100 revolutions per minute (rpm). After incubation, the suspension was passed through two layers of sterile cheesecloth to obtain only fungal spores. The filtrate was centrifuged at 3 220 relative centrifugal force (rcf) for 10 min before discarding the supernatant. Spores were washed twice by adding 20-30 mL sterile deionised $H_2O$, shaken, and again centrifuged at 3 220 rcf for 10 min. The supernatant was discarded after each wash and spores were finally re-suspended in 20-30 mL sterile deionised $H_2O$. The spore concentration was determined using a haemocytometer and adjusted to a final concentration of $2 \times 10^6$ spores $mL^{-1}$. One mL of the spore suspension was used to inoculate the primary ears via the silk channel (Reid *et al*., 1999). The control field plot of each replicate or control plants in the greenhouse trial were inoculated with 1 mL sterile deionised $H_2O$.

**Grain processing**

The primary ear of the maize inbred lines, planted in a greenhouse, was manually harvested and flash frozen in liquid nitrogen. The kernels were removed, course-milled using a sterile mortar and pestle, and subsequently fine-milled using the IKA A11 basic analytical mill (IKA laboratory technology, Staufen, Germany). The resultant flour was stored at -80ºC until analysis.

The primary ear of each hybrid planted in the field at Potchefstroom and Vaalharts was harvested at physiological maturity and pooled per row with fungal- and water-inoculated rows kept separately. Ears were threshed, and the grain was pooled. A 250-g sample of kernels per field replicate was stored at -20ºC, while an additional 50 g was stored at -80ºC for further

analyses. Kernels stored at -20ºC were course milled and subsequently milled to flour using a Phillips® juice blender (Philips, Amsterdam, Netherlands). The flour was sifted using a 1-mm sieve to obtain a uniform particle size and stored again at -20ºC until analysis. Kernels stored at -80ºC were milled as in the inbred line trial described above.

**Structural trait assessments**

Silk length, husk coverage, kernel hardness and pericarp thickness were assessed for both field and greenhouse trials unless stated otherwise.

*Silk length and husk coverage*

The silk length of maize was only evaluated for the cultivar trials. Silk length was measured at anthesis. For each cultivar, the silks of 25 primary ears were measured for each field plot. Measurements were taken from the point of silk emergence from the husk leaves to the tip of the silks and rounded off to the nearest half cm. Husk coverage was visually scored as either open or closed, depending on the visibility of the maize ear. For the inbred lines, husk coverage was scored at anthesis and harvest for each biological replicate. For each cultivar, 25 primary ears were scored per field plot. Husk coverage was scored at anthesis and reassessed at harvest.

*Kernel hardness*

Kernel hardness was assessed using two methods: Hundred-kernel mass (HKM) (Guelpa *et al*., 2015) and near-infrared (NIR) spectroscopy (Downey *et al*., 1986). HKM was determined by counting 100 intact kernels for each field (cultivars) or biological (inbred lines) replicate and weighed in grams.

Kernel hardness assessment by NIR spectroscopy was performed using the NIRFlex N-500 Frontier Transformed (FT-NIR) spectrophotometer (BÜCHI Labortechnik GmbH, Flawil, Switzerland) and NIR LabWare version 3.0 software (BÜCHI Labortechnik GmbH, Flawil, Switzerland). Samples were placed in a glass Petri plate and evaluated in the diffuse reflectance mode (Fig.1). Samples were subjected to NIR light at a resolution of 32 $cm^{-1}$ from 1 100 to 2 500 nm. Absorbance measurements was calculated by the NIR LabWare software based on the light reflected. Absorbance measurements (1 $R^{-1}$) at 2 230 nm were used in a formula proposed by Downey *et al*. (1986) to obtain index values for hardness as follows, hardness = $\alpha + b$ (log 1 $R^{-1}$), where $\alpha$ = -40 and $b$ = 100. For the inbred line trial, kernel hardness via NIR spectroscopy was assessed for flour samples and the cultivar trials was assessed using kernel and flour samples.

*Pericarp thickness*

The thickness of the kernel pericarp was measured using the ZEISS Stemi 508 Greenough Stereo Microscope (Carl Zeiss AG, Oberkochen, Germany) and visualised with the Axiocam ERc 5s camera (Carl Zeiss AG). Three intact kernels were soaked for 4 hours in sterile deionised $H_2O$ and cut longitudinally from the dent (Fig. 2) to the tip cap using a sterile scalpel. One half of each kernel was used to measure the thickness of the pericarp with two measurements recorded per kernel, one from the middle to lower region of the kernel and one from the top (dent) (Fig. 2). Three kernels were assessed for each field (cultivar trial) or biological (inbred line trial) replicate with the measurements recorded in mm. Measurements were obtained using the Zeiss Labscope mobile application (Carl Zeiss AG).

**Physico-chemical properties assessments**

Physico-chemical properties including moisture content, kernel pH, carbon and nitrogen content as well as phenolic acid content were assessed for both field and greenhouse trials unless stated otherwise.

*Moisture content and kernel pH*

The moisture content of maize grain was measured directly after harvest. For the inbred line trial, 5 to 10 kernels were used with three technical replications performed for each biological replicate. The moisture content was determined using a G7 Delmorst Moisture Meter (Delmorst Instrument Co., New Jersey, USA) at a temperature of 23-25ºC. For the cultivar trial, the moisture content of kernels was measured using the Draminkski TwistGrain moisture meter (Draminski Electronics, Olsztyn Poland). The moisture content was determined as a percentage at 20-25ºC. About 25 g of intact kernels were used per measurement and three observations taken for each field plot.

The pH of maize grain was measured using the Jenway 3510 pH meter (Bibby Scientific Limited, Staffordshire, UK). The pH was assessed by mixing 500 mg of finely milled flour with 5 mL of sterile deionised $H_2O$. Three technical replications for each field (cultivar trial) or biological (inbred line trial) replicate was assessed.

*Carbon and nitrogen analysis*

The total soluble nitrogen and carbon in kernels was assessed using the TruSpec® Micro (LECO Corporation, Michigan, USA) at the Central Analytical Facility (CAF) at Stellenbosch University. Carbon and nitrogen content were assessed by Vario EL Cube elemental analysis. Ten mg of dried, homogeneous maize flour was placed into an aluminium foil weighing boat and the sample was subjected to combustion at 1050ºC. The combustion took place within a

column filled with tungsten oxide ($WO_3$) and augmented with oxygen. The elements within the sample were bound to form the following gases: carbon dioxide, $H_2O$, nitrogen and nitrogen oxides as well as sulphur dioxide and sulphur trioxide. These gases were carried to the absorption columns where it was desorbed and quantified.

*Phenolic acid extractions and quantification*

Milled flour (1 g) was washed twice using 5 mL of hexane in a 50-mL Falcon tube (Merck, Darmstadt, Germany) by mixing the sample on an orbital shaker (Camlab, Cambridge, UK) at 200 rpm for 10 min at 25ºC before centrifuging at 3 220 rcf for 5 min. After centrifuging, the supernatant was discarded, and the samples allowed to dry in the fume hood for 1 hr at room temperature. Free phenolic compounds were extracted by adding 10 mL of methanol/water (80:20 vol/vol) (ROMIL Pure Chemistry Ltd., Cambridge, UK) using a 25-mL dispenser (Dragon Laboratory Instruments Ltd., Beijing, China). The mixture was placed in a shaking-incubator (Labcon™, CA, USA) at 120-150 rpm for 30 min at 25ºC, and thereafter centrifuged at 3 220 rcf for 10 min at 25ºC. Eight mL of the supernatant was collected in a new 15-mL Falcon tube, while the compressed flour was allowed to dry overnight in the fume hood for the extraction of bound phenolic acids. The supernatant was concentrated to 4 mL under a nitrogen stream before filling up the volume to 10 mL using sterile, deionised $H_2O$. Samples were acidified to a pH of 2.0-3.0 using between 110-120 µL 1 N HCl. The mixture was transferred to a new 50-mL Falcon tube and phenolic acids extracted by adding 10 mL of ethyl acetate (Sigma-Aldrich, Missouri, USA). The mixture was shaken on an orbital shaker (Camlab) at 150 rpm for 5 min at room temperature before being centrifuged at 3 220 rcf for 5 min. Eight mL of the supernatant was transferred to a new 15-mL Falcon tube. Samples were dried completely using a nitrogen stream at about 40ºC and stored at -20ºC for 1-2 weeks before analysis.

Flour pellets (100 mg) from the free phenolic acid extraction was subjected to alkaline hydrolysis using 4 mL of 2 N NaOH before shake-incubation in a dark nitrogen atmosphere for 2 hrs. The hydrolysis was stopped by acidifying the mixture using 10.2 N HCl until it reached a pH of 2-3. The extraction of phenolic acids was done by adding 5 mL ethyl acetate, shaking at room temperature on an orbital shaker (Camlab) at 150 rpm for 5 min, and centrifuging at 3 220 rcf for 5 min before transferring 4 mL of the supernatant into a new 15-mL Falcon tube. The extraction of bound phenolic acids was repeated and reduced to dryness using a nitrogen stream at about 40ºC. The samples were stored at -20ºC for 1-2 weeks before analysis. The precipitated samples were reconstituted in 200 µL of 50% methanol and underwent a 50% dilution before analysis.

Phenolic acids quantified represent the major phenolic acids found in the maize kernel pericarp layer (Sampietro *et al*. 2013; Das and Singh, 2016; Salinas-Moreno *et al*., 2017). The

quantification procedure employed an external standard dilution series using purified commercial products trans-ferulic, caffeic, ρ-coumaric, sinapic and ρ-hydroxybenzoic acids (Sigma-Aldrich). The standard curve consisted of a 10-point dilution series ranging from 0.0125–200 mg $kg^{-1}$. Quantification was performed at CAF, Stellenbosch University, using a Synapt G2 quadruple time of flight (Q-TOF) mass spectrometer (Waters, Milford, USA) with a chromatograph of Waters Ultra pressure liquid and a photo-diode array detection. Separation was accomplished using the High Strength Silica technology T3 operated in a negative electrospray ionisation mode (Waters, Milford, USA). The spectrometer was operated using nitrogen at 650 L $hr^{-1}$ at 275ºC for desolvation gas and temperature respectively, including a cone voltage of 15 V. MassLynx 4.1 software, allowed for data acquisition, consisted of a low energy scan (6V) to a high energy scan ranging from 150-1500 and 40-1500 m $z^{-1}$, respectively.

**Gene expression of defence-related genes**

*RNA isolation*

Total RNA was isolated as described by Wang *et al*. (2011). Flour, stored at -80ºC (0.2 g) was combined with 400 µL of TRIS-HCL pH 9 (Merck, Darmstadt, Germany), vortexed, and incubated at room temperature for 10 min. Following this, 20 µL of sodium dodecyl sulfate (SDS) (Melford, Ipswich, UK) was added and incubated once again at room temperature for 5 min. The suspension was centrifuged at 10 600 rcf for 10 min at 4ºC. From the resultant supernatant, 200 µL was taken and added to 400 µL of TRIzol Reagent (Sigma-Aldrich) before being vortexed and incubated at room temperature for 10 min. Chloroform (120 µL) was added before vortexing and subsequent centrifuging at 10 600 rcf for 10 min at 4ºC. The supernatant (300 µL) was added to 300 µL of isopropanol (Sigma-Aldrich) and thoroughly mixed by pipetting. Precipitation was performed at -20ºC for 20-30 min, followed by centrifugation at 12 500 rcf for 10 min at 4ºC. The resultant supernatant was discarded and 400 µL of 0.1% diethylpyrocarbonate (DEPC) (Sigma-Aldrich) was added to the pellet. Additionally, 400 µL of 1:1 phenol:chloroform was added, immediately mixed by pipetting, and the suspension was centrifuged at 10 600 rcf for 10 min at 4ºC. The supernatant (200 µL) was added to chloroform (200 µL) and centrifuged as previously described. The supernatant (100 µL) was added to a mixture containing 200 µL of 100% ethanol (Sigma-Aldrich) with 10 µL 3 N sodium acetate ($C_2H_3NaO_2$) (Sigma-Aldrich) and thoroughly mixed by pipetting. Another precipitation step was performed at -80ºC for 30-60 min before centrifuging at 12 500 rcf for 20 min at 4ºC. The supernatant was discarded, and the precipitate was washed twice with 500 µL of 70% ethanol and centrifuged at 10 600 rcf for 10 min at 4ºC, before the pellet was dried at room temperature for 10 min.

The RNA was treated with RNase-free DNase set (QIAGEN, Hilden Germany) according to the manufacturer's recommendations, and the RNA concentration determined using a ND-1000 NanoDrop Spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa). The purity of the RNA was determined by the 260/280 ratio where ~2.0 is seen as pure RNA and the 260/230 ratio where a value of 2.0-2.2 is considered generally free of contaminants. RNA samples were stored at -80ºC until analysis.

*cDNA synthesis and Reverse transcription quantitative PCR*

Complementary DNA (cDNA) was synthesised using the iScript cDNA synthesis kit (Bio-Rad Laboratories, USA). Samples were prepared according to the manufacturer's protocol, using 15 µL of RNA as template, 4 µL of iScript mix and 1 µL of the reverse transcriptase enzyme to achieve a total volume of 20 µL. The assay was performed using a thermocycler with run conditions as follows: 25ºC for 5 min, 46ºC for 20 min, 95ºC for 1 min followed by a holding temperature of 4ºC.

Reverse transcription quantitative PCR (RT-qPCR) was performed to quantify the relative expression of peroxidase (5'-GCACAAGGTCCTGTTCGTCT-3' and 5'-TTTCCCTGATCTCTCCCTCA-3'), *PR1* (5'-GAACTCGCCGCAGGACTAC-3' and 5'-GAGCCCCAGAAGAGGTTCTC-3') and *PR5* (5'-GTCATCGACGGCTACAACCT-3' and 5'-GGGCAGAAGGTGACTTGGTA-3') in the fungus-inoculated and water inoculated kernels using the primers reported by Lanubile *et al.* (2010). The elongation factor (*EF*)-1α was used as reference gene. Validation experiments were performed to show that efficiencies of the target genes and reference gene amplifications were equal (Bustin *et al.*, 2009). This was done by preparing standard curves for all the genes assessed using a 2X dilution series of pooled cDNA diluted in nuclease-free $H_2O$. The RT-qPCR reactions were carried out on the CFX96™ Real-Time system (Bio-Rad) according to the method described by Lanubile *et al.* (2010). The reactions contained 2 × iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 10 mM of each primer, and 20 ng of template cDNA. The run conditions were as follows: 95°C for 3 min and 44 cycles at 95°C 10 s, 60°C 25 s. A melt curve analysis ranging from 60-95°C was included in each run to ensure amplicon specificity. For the inbred line trial, biological replicates were analysed separately, with three technical replications. Cultivar field replications were pooled, and three technical replicates were performed for each cultivar. Template-free samples were included in each run that served as the no template control.

The relative expression of the defence-related genes was calculated for the fungus-inoculated and control samples using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where $\Delta C_t$ is the threshold cycle value difference between the target and reference gene of each reaction. The standard deviation of the $C_t$ values between replicates was less than 5%.

**Infection indicators**

Disease severity, fungal content and fumonisin contamination was assessed as infection indicators and were performed for both the field and greenhouse trials unless stated otherwise.

*Visual disease rating*

At harvest, the cultivar trial was assessed for visual disease severity. Severity assessments were performed on an arbitrary continuous scale from 1-10, with 1 being no visual symptoms and 10 being very severe. The primary ears of 15 plants were assessed for each field plot and expressed as a percentage. Visual disease rating was only scored for cultivar trial (field trials).

*Fumonisin extraction and quantification*

Fumonisins were extracted from maize flour using a methanol/water (70:30 vol/vol) extraction buffer according to Rose *et al.* (2016). A flour sample of 5 g was suspended in 20 mL of extraction buffer. Samples were placed in a shaking-incubator (Labcon™) at 200 rpm for 30 min at 25ºC and subsequently centrifuged at 500 rcf for 10 min at 4ºC. Of the supernatant, 2 mL was transferred to a microcentrifuge tube using a sterile syringe and passed through a 0.22 µm regenerated cellulose syringe filter (Bonna-Agela Technologies Inc., Tianjin, China). The extract was left overnight at 4ºC to precipitate and centrifuged at 16 300 rcf for 10 min. Of the supernatant, 1.5 mL was transferred to a glass vial (Bonna-Agela Technologies Inc.) and submitted to CAF, Stellenbosch University, for LC-MS/MS. Fumonisin standards $FB_1$, $FB_2$ and $FB_3$ was prepared as described by Small *et al*. (2012). A 6-point standard curve was set up ranging from 0,05 to 20,16 mg $kg^{-1}$ for $FB_1$ and $FB_2$ and 0,005 to 2,08 mg $kg^{-1}$ for $FB_3$.

*Quantitative PCR of Fusarium verticillioides in maize kernels*

Genomic DNA of grain samples and *F. verticillioides* MRC 826 mycelia was extracted according to Boutigny *et al*. (2012). Flour samples (2 g) was suspended in 10 mL CTAB/PVP extraction buffer (1.4 N NaCl, 0.1 N Tris/EDTA and 1% Polyvinylpyrrolidone (PVP) pH8) and 40 µL proteinase K (10 mg $mL^{-1}$) (Sigma-Aldrich). The samples were placed in a shaking-incubator (Labcon™) for 2 hrs shaking at 200 rpm at 65ºC. Samples were centrifuged for 10 min at 3 220 rcf at 25ºC before transferring 1 mL of the supernatant to a new microcentrifuge tube. RNA treatment was performed by adding 30 µL RNase A (10 mg $mL^{-1}$) (QIAGEN) and incubating samples in a water bath at 65ºC for 15 min. The samples were centrifuged for 10 min at 16 300 rcf and the supernatant (400 µL) transferred to a new microcentrifuge tube. DNA isolations from fungal material were subjected to additional purification steps (Boutigny *et al*., 2012). DNA isolation was completed using the DNeasy® Plant Mini Kit (QIAGEN) according to the manufacturer's recommendations commencing from step 9 where P3 buffer (130 µL) is

added and the mixture incubated on ice to allow for precipitation of cellular proteins, polysaccharides.

For the preparation of standard curves for quantitative PCR (qPCR), DNA from pathogen-free maize kernels and *F. verticillioides* MRC 826 was used. A 5-point matrix-matched dilution series was created with the fungal DNA diluted in pathogen-free maize DNA (10 ng $\mu L^{-1}$). The quantity of *F. verticillioides* DNA was determined by using primers Fver356 forward/Fver412 reverse (Nicolaisen *et al*., 2009) as described by Boutigny *et al*. (2012). Quantification was performed using the Rotor-Gene™ 6000 (Corbett Life Science, Mortlake, Australia). qPCR assays were carried out in 25-µL reaction volumes consisting of SensiMix™ SYBR® No-ROX Kit (Bioline, London, UK), 200 nM of each primer, and 2 µL of template DNA (10 ng $\mu L^{-1}$). PCR conditions was as follows: 95ºC for 10 min, followed by 35 cycles of 95ºC for 15 sec, 62ºC for 15 sec, 72ºC for 15 sec and a melting curve analysis from 72ºC to 95ºC, rising by 1ºC each step. The standard curve was created using 3 replicates of each dilution and included a no template control (NTC). The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines were taken into account as criteria for acceptance with a correlation coefficient ($R^2$) > 0.98, a slope (M)-value of between -3.2 and -3.4, and a reaction efficiency (E-value) of 0.98-1.05 (Bustin *et al*., 2009).

For quantification of *F. verticillioides* in the maize kernels, qPCR assays were performed in duplicate for each replicate. The melting temperature ($T_m$) of the amplicons was assessed to confirm analytical specificity. The standard deviation (SD) of the $C_t$ values for a sample was below 0.5. Standard DNA (16 times dilution) was included in the qPCR assay and analysed in triplicate. Using the matrix specific standard curves, the Ct values of sampled evaluated were transformed into DNA concentrations using the Rotor-Gene™ 2.0.2.4 software.

**Data analysis**

One-way analysis of variance (ANOVA) was performed to assess the response of inbred lines and cultivars for the parameters evaluated in this study. Where variation deviated from normality, log-transformation was performed to improve homogeneity. For the cultivar trial, ANOVA revealed significant differences between localities and, therefore, ANOVA was then performed per locality. Pearson's correlation was performed for each locality to determine correlations between resistance characteristics and infection indicators. Multifactor analysis (MFA) was assessed for all trials to determine whether significant correlations exist between plant traits and the infection indicators. For the MFA structural characteristics, physico-chemical properties and genetic responses were grouped and compared to the group of infection indicators. Partial least squares (PLS) regression was performed to test correlations between individual characteristics and the infection indicators group. Within these multivariate analyses, both the log-transformed and non-log-transformed data was included.

## RESULTS

One-way analysis of variance (ANOVA) was performed on the inbred line trial (Tables 3-5). When treatment variables were significantly different, inbred line by treatment interaction was assessed. For the cultivar trial, one-way ANOVA was initially performed on the combined datasets of the two field localities. Significant variation was found between the two localities even following analyses using a weighted ANOVA approach. Therefore, ANOVA was then performed for each locality and is presented as such (Tables 6-11). Results of *F. verticillioides*-inoculated samples are reported, unless otherwise stated.

### Inbred line trial

*Infection indicators*

The fumonisin levels of the fungal-inoculated grain of CB 222 (456.1 mg $kg^{-1}$) and R2565y (829.6 mg $kg^{-1}$) was significantly higher than all other samples recorded (Table 3).

Similarly, the fungal-inoculated grain of CB 222 (8.21 ng $\mu L^{-1}$) and R2565y (11.16 ng $\mu L^{-1}$) had the highest levels of fungal target DNA measured (Table 3).

*Phenotypic characteristics*

The ears of all inbred lines were closed by husk leaves; with the exception of inbred line CB 222 that had one open ear (data not shown). Kernel hardness, assessed on flour samples for inbred lines, indicated that the resistant line CML 390 (51.8) had the highest hardness index and did not differ significantly from CML 444 (39.8). Conversely, fumonisin-resistant line CB 222 (19.0) had the lowest hardness index (Table 4). Resistant line CML 390 (0.137 mm) had the thickest pericarp layer and it differed significantly from susceptible line R2565y (0.115 mm) and resistant line CML 444 (0.097 mm) which had the thinnest pericarp layer (Table 4). Line R2565y (51.0 g) had a significantly greater HKM than the other inbred lines, whereas CML 444 (26.3 g) had the lowest HKM.

*Physico-chemical*

The moisture content of fungal-inoculated grain from resistant line CML 390 (23.4%) was significantly higher compared to all other samples evaluated, with the exception of susceptible line R2565y (Inoculated; 22.8%). Conversely, resistant line CML 444 (Inoculated; 13.7%) had the lowest moisture content and did not differ significantly from water-inoculated grain of CML 444 (15.5%) (Table 5). The fungal-inoculated grain of line CML 390 was the least acidic (pH 6.7) and only differed significantly from the water-inoculated grain of line CB 222 (pH 6.3).

Line CB 222 (Inoculated; 138.2 mg $kg^{-1}$) had the greatest levels of free phenolic acids, not differing significantly from CB 222 (Control; 57.2 mg $kg^{-1}$) and R2565y (Control; 60.5 mg $kg^{-1}$) (Table 5; Fig. 3). CML 444 (Inoculated; 8.6 mg $kg^{-1}$) had the lowest levels of free phenolic acids and did not differ statistically from several other samples, including CML 390 (Inoculated; 14.9 mg $kg^{-1}$; control; 17.7 mg $kg^{-1}$) and R2565y (Inoculated; 17.3 mg $kg^{-1}$). Bound phenolic acids was the most abundant in water-inoculated grain of line CML 444 (232,1 mg $kg^{-1}$) and did not differ statistically from CML 390 water- (155.8 mg $kg^{-1}$) and- fungal-inoculated (190.2 mg $kg^{-1}$) grain (Table 5; Fig. 4). The water-inoculated grain of line CML 444 (244.3 mg $kg^{-1}$) also had the highest levels of total phenolic acids, not differing significantly from several other samples, including CML 390 (Inoculated; 205.0 mg $kg^{-1}$) and CB 222 (Inoculated; 232.8 mg $kg^{-1}$). R2565y (Inoculated; 73.7 mg $kg^{-1}$) had the lowest levels, not differing significantly from several others, including CML 444 (Inoculated; 100.5 mg $kg^{-1}$) and CB 222 (Control; 105.6 mg $kg^{-1}$).

The fungal-inoculated grain of CML 444 (2.3%) had the highest total nitrogen percentage, not differing significantly from CML 444 (Control; 2.1%) (Table 5). The lowest nitrogen percentage was observed in R2565y (Control; 1.3%) and did not differ significantly from R2565y (Inoculated; 1.6%) and CML 390 (Inoculated; 1.7%). Line CB 222 (Control; 39.2%) contained the highest carbon percentage and only differed significantly from line R2565y (Control; 28.1% and Inoculated; 31.5%). The water-inoculated grain of CB 222 and R2565y (22.3) had the greatest C/N ratio and only differed significantly from CML 444 (Inoculated; 16.9) and (Control; 17.7) (Table 5).

*Genetic response*

The relative expression of *peroxidase* was the highest in water-inoculated grain of susceptible line R2565y (9.1 fold change) and only differed significantly from the water- (0.11 fold change) and- fungal-inoculated (0.07 fold change) grain of resistant line CML 390 (Fig. 9). The relative expression of *PR1* was greatest in fumonisin-resistant line CB 222 (fungal-inoculated; 0.60 fold change) which did not differ significantly from a few other samples including the fungal- (0.04 fold change) and- water-inoculated (0.14 fold change) grain of R2565y (Fig. 10). The water-inoculated grain of CML 390 had the lowest expression (<0.00) similar to both fungal- and- water-inoculated grain of CML 444 (0.001 fold change). The water-inoculated sample of R2565y (2.98 fold change) had the highest relative expression of *PR5* but did not differ significantly from R2565y (fungal-inoculated; 2.56 fold change) and CB 222 (fungal-inoculated; 1.67 fold change) (Fig. 11). The lowest expression of *PR5* was seen in CML 390

(water-inoculated; 0.002 fold change), not differing significantly from CB 222 (water-inoculated; 0.04) and CML 444 (fungal-inoculated; 0.01 fold change).

**Cultivar trial**

*Infection indicators*

Disease severity in Potchefstroom (17.6%) was significantly lower than levels in Vaalharts (26.8%). In Potchefstroom, FER severity of cultivar 4 (12.7%) was the lowest but only differed significantly from three other cultivars namely cultivar 10 (28.4%), 9 (20.7%) and 7 (20.7%) (Table 6). No significant differences in total fumonisin levels between cultivars were determined in grain inoculated with *F. verticillioides* (Table 6). There was, however, significantly more fumonisins in cultivar 15 (33.6 mg $kg^{-1}$) 3 (25.0 mg $kg^{-1}$) and 8 (23.0 mg $kg^{-1}$) in the water-inoculated grain. Similarly, fungal target DNA levels in fungal-inoculated grain were not significantly different between cultivars (Table 6). Slightly more variation was observed in the water-inoculated grain of cultivar 3 (0.013 ng $\mu L^{-1}$) and 15 (0.009 ng $\mu L^{-1}$) having the highest levels of fungal target DNA (Table 6).

In Vaalharts cultivars 1 (15.1%) and 2 (16.4%) had significantly lower FER severity lower disease severity than all other cultivars evaluated (Table 7). Cultivar 11 (35.1%) had significantly higher disease symptoms when compared to other cultivars and did not differ significantly from several other cultivars including cultivar 12 (30.4%) and 4 (30.2%). Cultivar 1 (0.5 mg $kg^{-1}$) contained significantly less fumonisins in fungal-inoculated grain compared to all other cultivars, except cultivars 2 (1.6 mg $kg^{-1}$) and 9 (6.2 mg $kg^{-1}$). Generally, control grain had higher levels of fumonisins compared to that measured in the fungal-inoculated samples. Cultivar 5 (594.2 mg $kg^{-1}$) had the highest levels of fumonisins in the control grain and was statistically similar to several other cultivars, including cultivars 10 (360.4 mg $kg^{-1}$), 11 (177.6 mg $kg^{-1}$) and 13 (481.5 mg $kg^{-1}$) (Table 7).

No fungal target DNA was detected in the fungal-inoculated grain of cultivar 1 and the content did not differ significantly from cultivars 2 (0.006 ng $\mu L^{-1}$) and 9 (0.025 ng $\mu L^{-1}$) (Table 7). Furthermore, the inoculated grain of cultivar 5 (0.311 ng $\mu L^{-1}$) had the highest levels of fungal target DNA and did not differ significantly from several other cultivars, including cultivars 10 (0.145 ng $\mu L^{-1}$), 11 (0.114 ng $\mu L^{-1}$) and 13 (0.174 ng $\mu L^{-1}$). Similarly, in the water-inoculated grain, no target DNA was detected in cultivar 9 and this did not differ significantly from cultivars 1 (0.01 ng $\mu L^{-1}$) and 2 (0.003 ng $\mu L^{-1}$). Cultivar 5 (0.243 ng $\mu L^{-1}$) had the highest levels in the control samples, not differing significantly from several other cultivars including cultivars 10 (0.121 ng $\mu L^{-1}$), 11 (0.091 ng $\mu L^{-1}$), and 13 (0.191 ng $\mu L^{-1}$) (Table 7).

*Phenotypic characteristics*

Cultivar 4 (11.9 cm) had the longest exposed silks and did not differ significantly from a number of other cultivars, including cultivars 5 (11.6 cm) and 3 (9.7 cm) when evaluated at Potchefstroom (Table 8). Cultivar 1 (7.6 cm) had the shortest exposed silks with the length measured being statistically similar to most of the other cultivars, including cultivars 15 (7.7 cm), 2 (9.5 cm) and 13 (9.5 cm). At anthesis cultivars 1 (0.0%) and 11 (0.0%) displayed no open ears and these did not differ statistically from other cultivars including cultivars 4 (2.7%) and 13 (1.3%) (Table 8). Cultivar 10 (56.6%) had the highest percentage exposed maize ears but it did not differ significantly from several other cultivars, including cultivars 6 (49.3%) and 9 (38.7%). At harvest, cultivar 11 (17.3%) still displayed the lowest percentage of open ears and did not differ significantly from several other cultivars including cultivars 1 (21.3%) and 4 (20.0%). Cultivars 12 (74.7%), 9 (70.7%) and 10 (70.4%) had the highest percentage of exposed maize ears at harvest. Little variation was observed between cultivars when the 100-kernel mass (HKM) was evaluated (Table 8). Cultivars 5 (31.2 g), 9 (31.8 g) and 10 (31.4 g) had the greatest mass, respectively, but only differed significantly from cultivar 1 (23.1 g).

In Potchefstroom, kernel hardness, based on flour samples, revealed cultivar 9 (12.1) had the highest hardness index, not differing significantly from several other cultivars, including cultivars 5 (11.1) and 7 (10.0) (Table 8). Cultivar 6 (5.3) had the lowest hardness index and did not differ significantly from numerous other cultivars, including cultivars 1 (5.8) and 4 (5.6). When hardness was assessed on whole kernel samples, cultivar 7 (51.1) had the greatest hardness index and was statistically similar to several other cultivars, including cultivars 8 (47.3) and 9 (46.5). Cultivar 6 (35.3) again had the lowest hardness index and did not differ significantly from other cultivars including cultivar 3 (39.7) and 4 (39.6). Cultivar 1 (0.103 mm) had the thinnest pericarp but only differed significantly from cultivar 2 (0.167 mm), 3 (0.149 mm), 10 (0.140 mm) and 11 (0.139 mm) that had the thickest pericarp.

Significant differences between cultivars were determined for the different structural characteristics evaluated at Vaalharts (Table 9). Cultivar 2 (9.6 cm) had the longest silks, however, its length did not differ significantly from several other cultivars, including cultivar 9 (9.1 cm) and 10 (9.2 cm). Cultivar 15 (5.7 cm) had the shortest exposed silks and did not differ from cultivars 6 (6.2 cm), 11 (6.7 cm) as well as 3 and 14 (6.8 cm).

At anthesis, cultivar 1 had no open ears and was statistically similar to cultivars 2 (1.3%), 5 (9.3%), 8 (12.0%) and 10 (16.0%) while cultivars 3 and 4 (76.0%) had the highest percentage of open ears (Table 9). At harvest, cultivar 1 (4.0%) still had significantly lower levels of open ears compared to all other cultivars except cultivar 10 (20.0%). Cultivar 14 (73.3%) had the highest percentage of ears not covered by husk leaves but this did not differ significantly from several other cultivars, including cultivars 4 (73.1%), 2 (66.7%) and 15 (56.0%).

Cultivar 14 (40.9 g) had the greatest HKM measured whereas cultivar 15 (25.2 g) had the lowest mass with cultivar 12 (26.8 g) not differing significantly from cultivar 15 (Table 9).

In Vaalharts, cultivar 14 (15.8) had the highest hardness index when flour samples were evaluated while 11 (56.6), 14 (50.8) and 1 (50.0) had amongst the highest hardness indices for kernels. Cultivar 8 (5.8) had the lowest hardness index and did not differ significantly from cultivars 3 (7.1) and 15 (8.5) (Flour samples). Cultivar 10 (40.0) had the lowest hardness index but only differed significantly from cultivars 1 (50.0), 14 (50.8), 13 (49.0) and 11 (56.6) (whole kernel samples).

The thinnest pericarp layer was recorded for cultivar 12 (0.094 mm) and it only differed significantly from a few cultivars including 7 (0.146 mm), 14 (0.143 mm), 2 (0.128 mm) and 1 (0.116 mm) (Table 9).

*Physico-chemical properties*

In Potchefstroom, cultivar 5 (pH 6.40) and 4 (pH 6.39) had the highest pH measured in fungal-inoculated grain (Table 10). The grain of cultivar 10 (pH 5.99) was the most acidic and did not differ statistically from several other cultivars, including cultivars 11 (pH 6.04) and 13 (pH 6.05). Cultivars 3 and 10 (11.6%) had the lowest grain moisture content when fungal-inoculated grain was evaluated. These cultivars, however, only had significantly lower moisture levels than cultivar 8 (12.6%), 9 (12.2%) and 11 (12.3%). Similarly, cultivar 10, 2, 13 and 14 (11.7) had the lowest grain moisture when water-inoculated grain was evaluated and only differed significantly from cultivar 9 (12.6) (Table 10).

In Potchefstroom, little variation was determined between cultivars when free, bound and the total phenolic acid content was evaluated (Table 10). Cultivar 10 (0.95 mg $kg^{-1}$) had the highest amount of free phenolic acids, however, it only differed significantly from cultivar 5 (0.01 mg $kg^{-1}$) and 8 (0.04 mg $kg^{-1}$) that contained the lowest levels of free phenolic acids quantified (Fig. 5). Cultivar 14 (0.22 mg $kg^{-1}$) had significantly higher levels of bound phenolic acids quantified when compared to all the cultivars evaluated except cultivars 5 (0.09 mg $kg^{-1}$), 1 (0.05 mg $kg^{-1}$) and 15 (0.04 mg $kg^{-1}$) (Fig. 6). No significant differences between cultivars could be determined when the total phenolic acid content of the fungal-inoculated grain were evaluated.

Total nitrogen was the highest in cultivar 10 (1.43%) and its content did not differ significantly from cultivars 13 (1.3%), 14 (1.3%), 7 (1.3%) and 3 (1.3%) (Table 10). Cultivar 11 (1.0%) had the lowest nitrogen level but did not differ significantly from several other cultivars including cultivars 6 (1.0%) and 9 (1.1%). Cultivar 1 (39.1%) contained the least amount of total carbon when compared to all the cultivars evaluated except cultivars 2 (38.9%) and 3 (38.9%). Cultivar 15 (42.2%) had the highest level of total carbon, however, this was statistically similar to most of the other cultivars evaluated. In terms of the carbon:nitrogen

ratio (C/N), cultivar 11 (44.2) had the highest ratio while cultivar 10 (29.5) and 3 (30.1) had the lowest ratio (Table 10).

In Vaalharts, cultivar 1 (pH 6.06) had the highest pH assessed in fungal-inoculated grain, statistically similar to cultivar 2 (pH 5.95) (Table 11). Cultivar 12 (pH 4.96) was the most acidic, not differing significantly from cultivars 11 (pH 5.07) and 13 (pH 4.97). Cultivar 10 (13.5%) had the greatest grain moisture content. It did not differ significantly from a number of other cultivars, including cultivars 9 (13.0%), 11 (13.0%) and 14 (13.1%). Alternatively, cultivar 8 had the lowest grain moisture content, and did not differ significantly from several other cultivars including cultivars 1 (12.3%) and 15 (12.3%) (Table 11).

The lowest free phenolic acid levels were recorded in cultivars 5 and 14 (0.02 mg $kg^{-1}$) and only differed significantly from cultivars 4 (2.14 mg $kg^{-1}$), 3 (0.92 mg $kg^{-1}$) and 8 (0.52 mg $kg^{-1}$) which had the highest free phenolic acid content (Table 11; Fig. 7). The greatest levels of phenolic acids recorded were bound phenolics in cultivars grown in Vaalharts (Fig. 8). Cultivar 12 (71.35 mg $kg^{-1}$) had the greatest levels, statistically similar to several other cultivars, including cultivar 15 (42.39 mg $kg^{-1}$). Cultivar 3 (0.02 mg $kg^{-1}$) had the lowest bound phenolic acid content, not differing significantly from several other cultivars including cultivars 1 (0.10 mg $kg^{-1}$) and 6 (0.05 mg $kg^{-1}$). Like bound phenolic acid content, cultivar 12 (71.42 mg $kg^{-1}$) had the greatest levels of total phenolic acids and did not differ significantly from several other cultivars, including cultivar 15 (42.5 mg $kg^{-1}$). Overall, cultivar 6 (0.10 mg $kg^{-1}$) had the lowest phenolic acid content, similar to cultivars 1 and 5 (0.24 mg $kg^{-1}$), 2 (0.30 mg $kg^{-1}$) and 7 (0.46 mg $kg^{-1}$) (Table 11).

Cultivar 7 (1.4%) contained the greatest amount of nitrogen and only differed significantly from cultivars 1 (1.1%), 3 (1.2%) and 8 (1.1%) which contained the lowest nitrogen levels (Table 11). Cultivar 7 (45.5%) had a significantly greater carbon content that all other cultivars. Cultivar 3 (41.6%) however, had the lowest total carbon percentage, not differing significantly from cultivars 5, 8 and 10 (42.0%). In terms of carbon:nitrogen ratio (C/N) cultivar 1 (40.2) had the greatest ratio, statistically similar to several other cultivars, including cultivar 8 (39.3). Cultivar 6 (29.3) had the lowest ratio, statistically similar to several other cultivars, including cultivar 7 (32.3).

*Genetic response in Vaalharts*

No gene expression analyses were performed on cultivars evaluated at Potchefstroom due to the low levels of infection indicators. Gene expression could not be obtained for cultivar 15 (Vaalharts). Relative *peroxidase* expression (fold change) in the fungal-inoculated grain was higher in cultivar 14 (0.297 fold change) than any other cultivar evaluated (Fig. 12). Alternatively, cultivars 1, 3, 6 (0.034 fold change) and 4 (0.043 fold change) had the lowest expression of *peroxidase*. In the water-inoculated grain, cultivar 14 (1.182 fold change) still

had the greatest expression (Fig. 12). Cultivars 1 (0.059 fold change) and 13 (0.062 fold change) had the lowest relative expression. In terms of the fungal-inoculated grain, expression of *PR5* was highest in cultivar in cultivar 5 (0.143 fold change), not differing significantly from cultivar 14 (0.137 fold change) (Fig. 13). In the water-inoculated grain, cultivars 11 (0.057 fold change) and 14 (0.055 fold change) had the lowest expression. There was no *PR5* expression in the water-inoculated grain of cultivar 4, other than that, cultivar 9 (0.001 fold change) had the lowest expression (Fig. 13).

**Correlations between individual phenotypic and physico-chemical characteristics as well as genetic responses with infection indicators**

Only correlations with a Pearson's correlation coefficient (r) of ≥ 0.60 were considered as noteworthy correlations. Fumonisins and fungal target DNA levels (r = 0.82) correlated when the inbred line trial data was analysed (Table 12). In Potchefstroom, there were significant positive correlations between fumonisin levels and fungal target DNA levels with both inoculated (r = 0.70) and uninoculated (r = 0.74) samples (Table 13). In Vaalharts, pH had an inverse correlation with infection indicators including visual disease severity (r = -0.73), total fumonisins (r = -0.72) and fungal target DNA (r = -0.75) of inoculated grain (Table 14). The expression of the *PR5* gene in inoculated samples had significant positive correlations with different infection indicators, including total fumonisins of uninoculated (r = 0.72) and inoculated (r = 0.71) grain samples as well as with the fungal target DNA levels of the inoculated grain (r = 0.69). Infection indicators including FER severity had significant correlations with other infection indicators including total fumonisins of inoculated samples (r = 0.69). Fumonisins and fungal target DNA levels of inoculated grain (r = 0.94) had a strong positive correlation (Table 14). A similar trend was seen in the uninoculated samples, where fumonisins and fungal target DNA levels (r = 0.89) had a strong positive correlation. The fumonisin levels measured in uninoculated grain was also significantly correlated to fumonisin levels measured in inoculated grain (r = 0.71) as well as fungal target DNA levels (r = 0.75) of inoculated grain (Table 14).

**Multivariate analysis**

*Inbred line trial*

Principle component analysis (PCA) of inbred lines showed a clear separation of resistant lines CML 390- and- 444 and fumonisin-resistant line CB 222- and- susceptible line R2565y based on the first principle component (F1) (Fig. 14). The biplot accounted for 49.8% of the total variation, where F1 explained 33.4% and F2 explained 16.4% of the variation. Most of the characteristics and infection indicators evaluated made a significant contribution to F1,

including HKM, free phenolic acids, *PR1* and *PR5.* According to the multifactor analysis (MFA), the biplot represented 49.8% of the variation observed with principle component 1 (F1) representing 35.0% and F2 representing 14.8% of the total variation. Some of the groups of characteristics, representing different levels of resistance, associated together such as infection indicators and genetic response. However, no correlation between the different groups and infection indicators, based on the squared Pearson correlation coefficient (RV), could be determined (Fig. 15). Phenotypic characteristics (RV = 0.25), physico-chemical properties (RV = 0.15) and genetic responses (RV = 0.31) did not correlate with infection indicators. Partial least squares (PLS) analysis, however, revealed that certain characteristics within the different groups had significant correlations to infection indicators (as a group) based on the variable importance on the projection (VIP) value (Fig. 16). Structural characteristics included HKM (VIP = 1.7) and kernel hardness (NIR; VIP = 1.1), physico-chemical properties included moisture content (VIP = 1.6) and levels of free phenolic acids (VIP = 1.2). All three genes assessed had significant variable importance's in the projection (*Peroxidase*: VIP = 1.2; *PR1:* VIP = 1.6; *PR5:* VIP = 1.8).

*Cultivar trial*

Principle component biplot of cultivars response supported ANOVA analysis with the first principal component (F1) separating most of the variables based on locality (Fig. 17). The biplot explained 57.1% of the variation, with F1 responsible for 46.9% and F2 accounting for 10.3% of the variation observed. Most variables made a significant contribution to variation on F1, including infection indicators, genetic variables as well as pH and moisture content. Based on F1, most variables were positively associated with samples in Vaalharts and negatively associated with samples in Potchefstroom, with the exception of pericarp thickness, silk length, kernel pH and C/N. Only free phenolic acids significantly separated samples on F2 (Fig. 17). MFA was only performed for Vaalharts. Even though most of the variables evaluated in this study were associated with each other, no correlations between the different groups of variables (levels of resistance), including structural characteristics (RV = 0.08), physico-chemical properties (RV = 0.21) or genetic responses (RV = 0.30), with infection indicators could be determined (Fig. 18). The biplot explained 41.9% of the variation observed where 24.1% of the variation was explained by F1 and 17.8% explained by F2. Although most of the variables were positively associated, there were no significant correlations between the groups of factors. The group of infection indicators were, however, strongly associated with each other (Fig. 18). Since MFA could not determine a clear correlation of the groups of variables, partial least squares regression (PLS-R) analysis was performed for the group of infection indicators vs individual resistance characteristics (Fig. 19). Certain characteristics correlated with infection indicators namely physico-chemical properties including pH (VIP =

2.3), N (VIP = 1.2) and C/N (VIP = 1.3), genetic response of *PR5* in inoculated grain (VIP = 2.2), *PR5* expression of the control grain (VIP = 1.9) as well as *peroxidase* activity in the inoculated grain (VIP = 1.2) (Fig. 19).

## DISCUSSION

Plant defence mechanisms including physical attributes of the maize plant and kernel structure represents a formidable barrier to *F. verticillioides* infection and the accumulation of its mycotoxins, fumonisins. In this study, structural, physico-chemical and genetic characteristics of maize, potentially contributing to resistance to *F. verticillioides*, were explored in previously characterised maize inbred lines and uncharacterised commercial cultivars. This study is the first to report on the relationship between factors, representing different levels of resistance, to FER and fumonisin accumulation.

Resistance to *F. verticillioides* by maize is multifactorial; however, no association between structural, physico-chemical and genetic characteristics, representing different levels of resistance; and traditional infection indicators could be determined. Infection indicators including field evaluation for resistance, fumonisin and/or fungal quantification is the gold standard for determining plant response to *F. verticillioides*/fumonisins, however, the process is time consuming and labour intensive (Köppen *et al*., 2010; Sampietro *et al*., 2010). Therefore, the association of maize characteristics, that can be assessed much earlier and faster, would facilitate more efficient identification of resistant material. Although the different levels of resistance did not correlate with infection indicators, some individual characteristics strongly correlated with these.

Defence-related genes, particularly pathogenesis-related (PR) genes were a good indicator of potential resistance to *F. verticillioides*, in this study. The association of genes, including *PR5* and *peroxidase,* and infection indicators was observed in the inbred line and cultivar trials, respectively. This suggests that the genetic potential of the genotype is a key component of resistance to FER and fumonisin accumulation. A positive correlation was found between *peroxidase* and fungal- and- fumonisin contamination in both trials. Likewise, *PR1* expression in the inbred line trial and *PR5* in both trials also had positive correlations with fungal- and- fumonisin contamination. Defence-related genes were strongly induced in fungal-inoculated grain of fumonisin-resistant line CB 222 while it was down-regulated in the susceptible line R2565y. This demonstrates the ability of the fungus to influence the expression of defence-related genes and the potential of the plant to defend itself.

Defence-related gene expression was low at harvest in the resistant cultivars. Resistant cultivars 1 and 9 had lower *peroxidase* expression levels than the moderately susceptible cultivar 14. Similarly, cultivars 1 and 9 also had lower *PR5* expression levels than susceptible

cultivars 5, 10 and the moderately susceptible cultivar 14. Similar results were obtained for inbred lines where the expression of *peroxidase*, *PR1* and *PR5* was greater in R2565y (susceptible line) and CB 222 (susceptible to FER only) than in the resistant lines CML 390 and CML 444. These results suggest that resistant genotypes could have other genes that contribute to its observed resistance. Moreover, low gene expression at harvest potentially indicates that *F. verticillioides* is no longer perceived as a threat, due to low levels, and thus the plant does not require the expression of defence-related genes at this point. There are a range of other plant defence genes including *PR* genes and *ROS* genes that have been identified in the *F. verticillioides*-maize pathosystem that could be responsible for the resistant phenotype observed (Lanubile *et al*., 2010; Van Zyl, 2018).

Defence-related genes have been shown to be associated with resistance to FER and fumonisin accumulation in maize (Lanubile *et al*., 2010; 2017; Maschietto *et al*., 2016, Van Zyl, 2018). Most of these studies focused on gene expression at initial stages of kernel development; however, the results of this study are supported by the findings of Van Zyl (2018) who determined the genetic response of maize inbred lines as late as 52 days after inoculation with *F. verticillioides*. This is the first study that quantified defence-related gene expression, amongst other factors, following harvest and confirms that greater expression of defence related genes in susceptible genotypes after *F. verticillioides* infection.

Physico-chemical factors including kernel pH and the carbon:nitrogen ratio (C/N) strongly influenced the suitability of the kernel microenvironment for fungal infection and fumonisin accumulation. The kernel pH had a significant inverse relationship with infection indicators in the cultivar trial. Previous studies, such as Flaherty *et al*. (2003) determined that acidic conditions were associated with fumonisin accumulation *in vitro*. The kernel pH is a vital aspect during *F. verticillioides* growth and fumonisin production. Under alkaline kernel conditions, fumonisin production is suppressed (Picot *et al*., 2011). In our study, maize cultivars in Vaalharts, had significantly more fungal and fumonisin contamination and lower grain pH as compared to Potchefstroom. Furthermore, studies show that *F. verticillioides* growth is associated with higher pH and C/N during the earlier stages of kernel development, and more acidic and lower C/N conditions to be associated with fumonisin accumulation (Jimenez *et al*., 2003; Duncan and Howard, 2010). Within this study FER severity, fumonisin contamination and fungal target DNA levels were all negatively associated with pH and C/N because infection indicators were assessed in mature maize kernels.

The role of phenolic acids in the defence against *F. verticillioides* was unclear in this study. Inbred lines showed a correlation between free phenolic acids and infection indicators; however, this was not observed in the cultivars. In a study by Ferrochio *et al*. (2013), low levels of ferulic acid were not associated with plant resistance. It was further suggested that for significant reduction of FER and fumonisin contamination to be achieved, an external

application of ferulic acid should be applied (Ferrochio *et al*., 2013). The results in this study, however, indicate no direct link between phenolic acid quantity and resistance to *F. verticillioides* or fumonisin accumulation in mature maize kernels. Phenolic compounds are not induced to inhibit *F. verticillioides* growth or fumonisin contamination in mature maize kernels (Cassiem, 2018). Other physico-chemical properties could also regulate fungal and fumonisin contamination in the grain, such as sugar and starch quantity, and should also be evaluated.

Kernel hardness and HKM correlated to infection indicators in the inbred line trial, however, when expanded to the cultivar trial, there was no correlation. This difference could be ascribed to the limited sample size of the inbred line trial, compared to the cultivar trial. Furthermore, structural characteristics and infection indicators, whether evaluated as a group or as individual characteristics, did not correlate. This could be due to the artificial inoculation employed that breeched certain structural barriers such as silk length and husk coverage. Nonetheless, there was also no strong correlation between infection indicators of uninoculated samples with structural characteristics. These results suggest that physical features are not good indicators of resistance to FER/fumonisins.

Commercial cultivars in this study showed varying degrees of resistance to FER and fumonisin accumulation. This was especially evident in Vaalharts where there was significant fungal and fumonisin contamination. FER and fumonisin accumulation occur most frequently in, dry conditions (Cao *et al*., 2014). Environmental conditions favoured disease development and fumonisin production in Vaalharts, as seen with the high levels of infection indicators in certain cultivars. The infection indicator results in Vaalharts, therefore suggest that cultivars 1, 2 and 9 have a high level of resistance to *F. verticillioides* and fumonisin accumulation. All three of these cutlivars are GM cultivars. Conversely, cultivars 5 (non-GM), 10 (GM) and 13 (non-GM) are considered as highly susceptible cultivars. This supports findings that commercial cultivars in South Africa display varying degrees of resistance to FER and fumonisin accumulation (Janse van Rensburg *et al*., 2015).

The water-inoculated grain samples were more contaminated with fumonisins than the *F. verticillioides*-inoculated samples in the cultivar trial in Vaalharts. The response of cultivars was, however consistent between the two plots further supporting the resistance status of the aforementioned cultivars. Field conditions present a variety of factors that could have contributed to this occurrence. Naturally-occuring fumonisin-producing isolates may have established better under the prevailing conditions compared to the isolates used for inoculation. Other sources of inoculum such as from seed, soil or airborne inoculum could also have contributed to the increased fumonisin levels in uninoculated grain (Munkvold, 2003). *Fusarium verticillioides* is the most abundant fumonisin-producing species in South Africa with different isolates possessing highly variable toxigenic potential and generally similar virulence

(Schoeman *et al*., 2018). Although this study showed strong correlations between FER symptoms, fungal growth and fumonisin contamination, certain isolates could be present in very low quantities, proving symptomless yet having a very high toxigenic potential (Miedaner *et al*., 2010; Schoeman *et al*., 2018).

In conclusion, the evaluation of physical, biochemical and genetic characteristics, revealed key associations with traditional infection indicators. The genetic response of maize genotypes to *F. verticillioides* infection was paramount for resistance to FER and fumonisin contamination. Furthermore, conditions within the kernel microenvironment such as pH and C/N ratio could serve as indicators of potential resistance. The results from this study, therefore, provide breeders with additional tools for more efficient selction of resistant material Multi-site, multi-year evaluation of the cultivars used in this study is warranted to determine the stability of their response to *F. verticillioides* infection. Finally, the expression of defence-related genes not only provided an indication of potential resistance but also provided more evidence for the molecular mechanisms governing resistance to *F. verticillioides*.

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**Table 1**. Maize inbred lines grown under greenhouse conditions at Stellenbosch University during the 2017/18 season.

| Inbred line | Resistant status[1] | Source[2] |
|---|---|---|
| **CB 222** | Susceptible to FER/ resistant to fumonisins | ARC-GCI- South Africa |
| **CML 390** | Resistant to FER/fumonisins | CIMMYT-Zimbabwe |
| **CML 444** | Resistant to FER/fumonisins | CIMMYT-Zimbabwe |
| **R2565y** | Susceptible to FER/fumonisins | ARC-GCI- South Africa |

[1]Resistance status according to Small *et al*. (2012), Rose *et al*. (2016) and Netshifhefhe *et al*. (2018).

[2]ARC-GCI – Agricultural Research Council-Grain Crop Institute, CIMMYT - International Maize and Wheat Improvement Centre.

FER – Fusarium ear rot.

Fumonisins – total of $FB_1$, $FB_2$ and $FB_3$.

**Table 2.** Fifteen commercial maize cultivars evaluated at Potchefstroom and Vaalharts during the 2016/17 season.

| Cultivar number | Cultivar name | Grain colour | GM/non-GM |
|---|---|---|---|
| 1 | DKC74-74BR | Yellow | GM |
| 2 | PAN6R-710BR | Yellow | GM |
| 3 | LS8526 | Yellow | Non-GM |
| 4 | KKS8410BR | Yellow | GM |
| 5 | PAN5A-182 | Yellow | Non-GM |
| 6 | IMP52-12R | Yellow | GM |
| 7 | BG3292 | Yellow | Non-GM |
| 8 | P2864WYR | White | GM |
| 9 | DKC78-79BR | White | GM |
| 10 | LS8539B | White | GM |
| 11 | IMP52-11R | White | GM |
| 12 | US9777 | White | Non-GM |
| 13 | US9711 | White | Non-GM |
| 14 | KKS4581BR | White | GM |
| 15 | P1659W | White | Non-GM |

GM – Genetically modified

Non-GM – Non-genetically modified

**Table 3**. Total fumonisins and *Fusarium verticillioides* target DNA accumulated in maize inbred lines during the 2017/18 season.

| Inbred line | Total fumonisins (mg $kg^{-1}$) * | | Target DNA (ng $\mu L^{-1}$) * | |
|---|---|---|---|---|
| | Inoculated | Control | Inoculated | Control |
| **CB 222** | 456.1 A | 1.8 B | 8.21 A | 0.21 B |
| **CML 390** | 35.9 B | 1.1 B | 0.61 B | 0.00 B |
| **CML 444** | 6.8 B | 12.3 B | 0.39 B | 0.48 B |
| **R2565y** | 829.6 A | 9.2 B | 11.16 A | 1.44 B |
| **Mean** | 332.1 | 6.1 | 5.1 | 0.5 |

Means followed by the same alphabetical letter in each column are not significantly different according to Fisher's LSD ($P \geq 0.05$).

*Mean recorded data followed by significant groupings based on log transformed mean.

**Table 4**. Structural characteristics of maize inbred lines evaluated during the 2017/18 season.

| Inbred line | Pericarp thickness (mm) | 100-kernel mass (g) | Kernel hardness* |
|---|---|---|---|
| **CB 222** | - | - | 19.0 C |
| **CML 390** | 0.137 A | 44.1 B | 51.8 A |
| **CML 444** | 0.097 C | 26.3 C | 39.8 AB |
| **R2565y** | 0.115 B | 51.0 A | 37.3 B |
| **Mean** | 0.116 | 40.5 | 37.0 |

Means followed by the same alphabetical letter in each column are not significantly different according to Fisher's LSD ($P \geq 0.05$).

-data not recorded.

* Determined by Near-infrared (NIR) spectroscopy.

**Table 5**. Physico-chemical properties of maize inbred lines evaluated during the 2017/18 season.

Means followed by the same alphabetical letter in each column are not significantly different according to Fisher's LSD (P≥0.05).

***Mean of raw data is presented and followed by significant groupings based on log transformed means.

| | CB 222 | | CML 390 | | CML 444 | | R2565y | | Mean |
|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Control** | **Inoculated** | **Control** | **Inoculated** | **Control** | **Inoculated** | **Control** | **Inoculated** | |
| **Moisture content (%)** | - | - | 19.6 B | 23.4 A | 15.5 C | 13.7 C | 19.8 B | 22.8 AB | 19.1 |
| **pH** | 6.3 B | 6.4 AB | 6.6 A | 6.7 A | 6.5 AB | 6.6 A | 6.5 AB | 6.5 AB | 6.5 |
| **Free phenolic acids (mg $kg^{-1}$) *** | 57.2 AB | 138.2 A | 17.7 CD | 14.9 D | 12.2 D | 8.6 D | 60.5 AC | 17.3 BD | 40.8 |
| **Bound phenolic acids (mg $kg^{-1}$) *** | 48.4 CD | 94.6 BC | 155.8 AB | 190.2 A | 232.1 A | 91.9 BD | 47.1 D | 56.4 CD | 114.6 |
| **Total phenolic acids (mg $kg^{-1}$) *** | 105.6 BD | 232.8 AB | 173.5 AC | 205.0 AB | 244.3 A | 100.5 CD | 107.6 BD | 73.7 D | 155.4 |
| **Total N (%)** | 1.8 BC | 1.9 AC | 1.9 BC | 1.7 BD | 2.1 AB | 2.3 A | 1.3 D | 1.6 CD | 1.8 |
| **Total C (%)** | 39.2 A | 37.8 A | 37.0 A | 34.2 AB | 37.0 A | 38.5 A | 28.1 C | 31.5 BC | 35.4 |
| **C/N** | 22.3 A | 19.7 AB | 20.3 AB | 20.4 AB | 17.7 B | 16.9 B | 22.3 A | 19.5 AB | 19.9 |

- Data not recorded.

**Table 6.** Fusarium ear rot severity, total fumonisins and *Fusarium verticillioides* target DNA content in 15 commercial maize cultivars evaluated at Potchefstroom during the 2016/17 season.

| Cultivar | | FER severity (%) | Total fumonisins (i) mg $kg^{-1}$* | Fumonisins (c) mg $kg^{-1}$* | Total target DNA (i) ng $uL^{-1}$* | Total target DNA (c) ng $uL^{-1}$* |
|---|---|---|---|---|---|---|
| **Number** | **Name** | | | | | |
| **1** | **DKC74_74BR** | 15.6 BC | 3.54 A | 1.3 BC | 0.007 A | 0.000 C |
| **2** | **PAN6R_710BR** | 16.9 BC | 1.19 A | 0.0 C | 0.000 A | 0.000 C |
| **3** | **LS8526** | 17.1 BC | 0.00 A | 25.0 A | 0.000 A | 0.013 A |
| **4** | **KKS8410BR** | 12.7 C | 0.00 A | 0.0 C | 0.000 A | 0.000 C |
| **5** | **PAN5A_182** | 14.9 BC | 0.01 A | 0.0 C | 0.000 A | 0.000 C |
| **6** | **IMP52_12R** | 17.3 BC | 6.50 A | 0.0 C | 0.005 A | 0.000 C |
| **7** | **BG3292** | 20.7 B | 6.09 A | 0.1 C | 0.004 A | 0.000 C |
| **8** | **P2864WYR** | 18.2 BC | 3.30 A | 23.0 AB | 0.007 A | 0.000 C |
| **9** | **DKC78_79BR** | 20.7 B | 3.13 A | 3.6 AC | 0.000 A | 0.000 C |
| **10** | **LS8539B** | 28.4 A | 0.22 A | 0.0 C | 0.004 A | 0.000 C |
| **11** | **IMP52_11R** | 16.2 BC | 9.63 A | 0.0 C | 0.010 A | 0.003 BC |
| **12** | **US9777** | 17.6 BC | 6.42 A | 0.2 C | 0.011 A | 0.000 C |
| **13** | **US9711** | 15.6 BC | 1.47 A | 0.0 C | 0.000 A | 0.000 C |
| **14** | **KKS4581BR** | 16.4 BC | 0.01 A | 0.0 C | 0.000 A | 0.000 C |
| **15** | **P1659W** | 15.3 BC | 0.39 A | 33.6 AC | 0.000 A | 0.009 AB |
| **Means** | | 17.6 | 2.8 | 5.8 | 0.0 | 0.0 |

FER – Fusarium ear rot. i – Inoculated (fungal-inoculated).

c – Control (water-inoculated).

FER severity calculated as the percentage of maize ears with visual symptoms represented by the mean disease severity of three field plots.

Means followed by the same alphabetical letter in each column are not significantly different according to Fisher's LSD ($P \geq 0.05$).

* Mean of raw data is presented and followed by significant groupings based on log transformed mean.

**Table 7.** Fusarium ear rot severity, total fumonisins and *Fusarium verticillioides* target DNA content in 15 commercial maize cultivars evaluated at Vaalharts during the 2016/17 season.

| Cultivar | | FER severity (%) | Total fumonisins (i) mg $kg^{-1}$* | Total fumonisins (c) mg $kg^{-1}$* | *Fusarium verticillioides target DNA* (i) ng $\mu L^{-1}$* | *Fusarium verticillioides target DNA* (c) ng $\mu L^{-1}$* |
|---|---|---|---|---|---|---|
| **Number** | **Name** | | | | | |
| **1** | **DKC74-74BR** | 15.1 D | 0.5 D | 0.1 F | 0.000 D | 0.006 DE |
| **2** | **PAN6R-710BR** | 16.4 D | 1.6 D | 0.2 F | 0.006 D | 0.003 E |
| **3** | **LS8526** | 28.9 AC | 18.1 C | 30.5 DE | 0.047 BC | 0.055 BC |
| **4** | **KKS8410BR** | 30.2 AB | 24.0 AC | 467.8 AB | 0.099 AB | 0.164 AB |
| **5** | **PAN5A-182** | 28.7 AC | 146.8 A | 594.2 A | 0.311 A | 0.243 A |
| **6** | **IMP52-12R** | 27.3 BC | 28.4 AC | 286.8 AC | 0.122 AB | 0.116 AC |
| **7** | **BG3292** | 29.1 AC | 64.2 AC | 127.9 BC | 0.124 AB | 0.089 BC |
| **8** | **P2864WYR** | 28.2 BC | 67.4 AC | 185.8 AC | 0.137 AB | 0.110 AC |
| **9** | **DKC78-79BR** | 23.3 C | 6.2 D | 1.4 EF | 0.025 CD | 0.000 E |
| **10** | **LS8539B** | 28.0 BC | 115.8 AB | 360.4 AC | 0.145 AB | 0.121 AC |
| **11** | **IMP52-11R** | 35.1 A | 84.7 AC | 177.6 AC | 0.114 AB | 0.091 AC |
| **12** | **US9777** | 30.4 AB | 52.8 AC | 97.2 CD | 0.111 AB | 0.061 CD |
| **13** | **US9711** | 28.0 BC | 73.7 AB | 481.5 AB | 0.174 A | 0.191 AB |
| **14** | **KKS4581BR** | 24.0 BC | 29.0 AC | 206.0 AC | 0.106 AB | 0.096 AC |
| **15** | **P1659W** | 28.4 AC | 20.7 BC | 76.0 CD | 0.050 BC | 0.062 BC |
| **Means** | | 26.8 | 48.9 | 206.2 | 0.1 | 0.1 |

FER – Fusarium ear rot.

i – Inoculated (fungal-inoculated).

c – Control (water-inoculated).

FER severity calculated as the percentage of maize ears with visual symptoms represented by the mean disease severity of three field plots.

Means followed by the same alphabetical letter in each column are not significantly different according to Fisher's LSD ($P \geq 0.05$).

* Mean of raw data is presented and followed by significant groupings based on log transformed mean.

**Table 8.** Structural characteristics of 15 commercial maize cultivars evaluated at Potchefstroom during the 2016/17 season.

| Cultivar | | Silk length (cm) | Husk coverage (% open) | | 100-kernel mass (g) | Kernel hardness* | | Pericarp thickness (mm) |
|---|---|---|---|---|---|---|---|---|
| Number | Name | | Anthesis | Harvest | | Flour | Kernels | |
| 1 | DKC74_74BR | 7.6 C | 0.0 C | 21.3 BC | 23.1 B | 5.8 E | 42.7 BC | 0.103 D |
| 2 | PAN6R_710BR | 9.5 AC | 26.7 AC | 45.3 AC | 25.9 AB | 6.9 CE | 39.9 BC | 0.167 A |
| 3 | LS8526 | 9.7 AC | 10.7 BC | 28.0 BC | 27.4 AB | 6.0 DE | 39.7 BC | 0.149 AB |
| 4 | KKS8410BR | 11.9 A | 2.7 C | 20.0 C | 28.7 AB | 5.6 E | 39.6 BC | 0.137 AD |
| 5 | PAN5A_182 | 11.6 AB | 5.3 BC | 37.3 AC | 31.2 A | 11.1 AB | 40.3 BC | 0.118 BD |
| 6 | IMP52_12R | 8.8 C | 49.3 AB | 61.3 AB | 27.8 AB | 5.3 E | 35.3 C | 0.113 BD |
| 7 | BG3292 | 9.1 BC | 14.7 AC | 49.3 AC | 28.2 AB | 10.0 AC | 51.1 A | 0.122 BD |
| 8 | P2864WYR | 8.7 C | 41.3 AC | 61.1 AB | 25.5 AB | 9.8 AD | 47.3 AB | 0.121 BD |
| 9 | DKC78_79BR | 8.1 C | 38.7 AC | 70.7 A | 31.8 A | 12.1 A | 46.5 AB | 0.129 BD |
| 10 | LS8539B | 9.3 AC | 56.6 A | 70.4 A | 31.4 A | 8.0 BE | 40.3 BC | 0.140 AC |
| 11 | IMP52_11R | 8.1 C | 0.0 C | 17.3 C | 30.1 AB | 6.4 CE | 43.4 AC | 0.139 AC |
| 12 | US9777 | 9.2 BC | 41.3 AC | 74.7 A | 26.9 AB | 6.6 CE | 40.6 BC | 0.131 AD |
| 13 | US9711 | 9.5 AC | 1.3 C | 34.7 AC | 24.5 AB | 9.8 AD | 40.7 BC | 0.127 BD |
| 14 | KKS4581BR | 9.1 BC | 10.7 BC | 34.7 AC | 28.0 AB | 7.8 BE | 41.9 BC | 0.125 BD |
| 15 | P1659W | 7.7 C | 16.0 AC | 40.0 AC | 28.2 AB | 7.1 CE | 43.7 AB | 0.110 CD |
| Mean | | 9.2 | 21.0 | 44.4 | 27.9 | 7.9 | 42.2 | 0.1 |

Means followed by the same alphabetical letter in each column are not significantly different according to Fisher's LSD ($P \geq 0.05$).

* Determined by Near-infrared (NIR) spectroscopy.

**Table 9.** Structural characteristics of 15 commercial maize cultivars evaluated at Vaalharts during the 2016/17 season.

| Cultivar | | Silk length (cm) | Husk coverage (% open) | | 100-kernel mass (g) | Kernel hardness* | | Pericarp thickness (mm) |
|---|---|---|---|---|---|---|---|---|
| Number | Name | | Anthesis | Harvest | | Flour | Kernels | |
| 1 | DKC74_74BR | 8.3 AB | 0.0 E | 4.0 F | 30.1 EG | 12.5 BD | 50.0 AC | 0.116 C |
| 2 | PAN6R_710BR | 9.6 A | 1.3 E | 66.7 A | 30.3 EF | 14.5 AB | 47.2 BD | 0.128 AC |
| 3 | LS8526 | 6.8 CE | 76.0 A | 44.0 BC | 31.9 CF | 7.1 FG | 47.7 BD | 0.114 CD |
| 4 | KKS8410BR | 8.4 AB | 76.0 A | 73.1 A | 32.5 CE | 11.4 CD | 46.8 BD | 0.108 CD |
| 5 | PAN5A_182 | 8.4 AB | 9.3 DE | 40.0 CD | 29.0 FG | 10.7 DE | 47.8 BC | 0.123 BC |
| 6 | IMP52_12R | 6.2 DE | 49.3 BC | 72.0 A | 32.0 CF | 12.2 BD | 48.1 BC | 0.110 CD |
| 7 | BG3292 | 8.2 AC | 21.1 BC | 45.3 BC | 34.8 BC | 11.3 CE | 48.5 BC | 0.146 A |
| 8 | P2864WYR | 8.5 AB | 12.0 DE | 24.0 DE | 37.3 B | 5.8 G | 42.8 CD | 0.114 CD |
| 9 | DKC78_79BR | 9.1 A | 54.7 AC | 56.0 AC | 37.4 B | 11.7 BD | 43.9 BD | 0.109 CD |
| 10 | LS8539B | 9.2 A | 16.0 DE | 20.0 EF | 34.2 BD | 10.4 DE | 40.0 D | 0.116 CD |
| 11 | IMP52_11R | 6.7 DE | 30.7 CD | 45.3 BC | 32.1 CF | 14.1 AC | 56.6 A | 0.107 CD |
| 12 | US9777 | 7.4 BD | 60.0 AB | 60.0 AB | 26.8 GH | 9.9 DF | 47.0 BD | 0.094 D |
| 13 | US9711 | 8.6 AB | 57.3 AB | 60.0 AB | 31.3 DF | 9.9 DF | 49.0 AC | 0.120 C |
| 14 | KKS4581BR | 6.8 CE | 49.3 BC | 73.3 A | 40.9 A | 15.8 A | 50.8 AB | 0.143 AB |
| 15 | P1659W | 5.7 E | 41.3 BC | 56.0 AC | 25.2 H | 8.5 EG | 47.5 BD | 0.114 CD |
| | Mean | 7.9 | 37.0 | 49.3 | 32.4 | 11.0 | 47.6 | 0.117 |

Means followed by the same alphabetical letter in each column are not significantly different according to Fisher's LSD ($P \geq 0.05$).

* Determined by Near infrared spectroscopy.

**Table 10**. Physico-chemical properties of 15 commercial maize cultivars evaluated at Potchefstroom during the 2016/17 season.

| Cultivar | | pH | | Moisture content (%) | | Free phenolic acids (mg kg$^{-1}$) * | | Bound phenolic acids (mg kg$^{-1}$) * | | Total phenolic acids (mg kg$^{-1}$) * | | Total nitrogen (%) | | Total carbon (%) | | C/N | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Number** | **Name** | | | | | | | | | | | | | | | | |
| **1** | **DKC74_74BR** | 6.34 | AC | 11.9 | BD | 0.09 | AB | 0.05 | AB | 0.14 | A | 1.1 | BD | 39.1 | B | 36.9 | AD |
| **2** | **PAN6R_710BR** | 6.32 | AD | 12.1 | AD | 0.11 | AB | 0.03 | B | 0.14 | A | 1.1 | BD | 38.9 | B | 35.3 | AD |
| **3** | **LS8526** | 6.37 | AB | 11.6 | D | 0.06 | AB | 0.02 | B | 0.08 | A | 1.3 | AC | 38.9 | B | 30.1 | D |
| **4** | **KKS8410BR** | 6.39 | A | 11.7 | CD | 0.11 | AB | 0.02 | B | 0.13 | A | 1.1 | BD | 41.2 | A | 37.0 | AD |
| **5** | **PAN5A_182** | 6.40 | A | 11.9 | BD | 0.01 | B | 0.09 | AB | 0.11 | A | 1.1 | BD | 42.0 | A | 37.1 | AD |
| **6** | **IMP52_12R** | 6.17 | AD | 12.1 | AD | 0.70 | AB | 0.06 | B | 0.76 | A | 1.0 | CD | 41.7 | A | 41.7 | AB |
| **7** | **BG3292** | 6.21 | AD | 12.0 | BD | 0.39 | AB | 0.01 | B | 0.40 | A | 1.3 | AC | 41.9 | A | 33.2 | BD |
| **8** | **P2864WYR** | 6.20 | DE | 12.6 | A | 0.04 | B | 0.02 | B | 0.06 | A | 1.1 | BD | 41.4 | A | 36.1 | AD |
| **9** | **DKC78_79BR** | 6.20 | AD | 12.2 | AC | 0.24 | AB | 0.02 | B | 0.26 | A | 1.1 | BD | 42.1 | A | 32.6 | BD |
| **10** | **LS8539B** | 5.99 | E | 11.6 | D | 0.95 | A | 0.06 | B | 1.00 | A | 1.4 | A | 41.9 | A | 29.5 | D |
| **11** | **IMP52_11R** | 6.04 | DE | 12.3 | AB | 0.76 | AB | 0.01 | B | 0.77 | A | 1.0 | D | 41.9 | A | 44.2 | A |
| **12** | **US9777** | 6.11 | CD | 11.9 | BD | 0.25 | AB | 0.02 | B | 0.27 | A | 1.1 | BD | 41.9 | A | 39.5 | AC |
| **13** | **US9711** | 6.15 | DE | 12.1 | AD | 0.33 | AB | 0.06 | B | 0.39 | A | 1.3 | AB | 42.0 | A | 31.6 | CD |
| **14** | **KKS4581BR** | 6.21 | DE | 12.0 | BD | 0.20 | AB | 0.22 | A | 0.42 | A | 1.3 | AC | 42.1 | A | 33.3 | BD |
| **15** | **P1659W** | 6.29 | BD | 12.0 | BD | 0.22 | AB | 0.04 | AB | 0.26 | A | 1.1 | BD | 42.2 | A | 38.2 | AD |
| | **Means** | 6.2 | | 12.0 | | 0.3 | | 0.0 | | 0.3 | | 1.2 | | 41.3 | | 35.7 | |

Means followed by the same alphabetical letter in each column are not significantly different according to Fisher's LSD ($P \geq 0.05$).

*Mean recorded data followed by significant groupings based on log transformed mean.

**Table 11**. Physico-chemical properties of 15 commercial maize cultivars evaluated at Vaalharts during the 2016/17 season.

| Cultivar | | pH | | Moisture content (%) | | Free phenolic acids (mg kg$^{-1}$) * | | Bound phenolic acids (mg kg$^{-1}$) * | | Total phenolic acids (mg kg$^{-1}$) * | | Total nitrogen (%) | | Total carbon (%) | | C/N | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Number** | **Name** | | | | | | | | | | | | | | | | |
| **1** | **DKC74_74BR** | 6.06 | A | 12.3 | CE | 0.14 | BC | 0.10 | CD | 0.24 | EF | 1.1 | C | 42.3 | BD | 40.2 | A |
| **2** | **PAN6R_710BR** | 5.95 | A | 12.7 | BD | 0.20 | AC | 0.10 | CD | 0.30 | EF | 1.2 | AC | 42.6 | BC | 37.0 | AD |
| **3** | **LS8526** | 5.40 | B | 12.5 | BE | 0.92 | A | 0.02 | D | 0.94 | DE | 1.1 | BC | 41.6 | E | 37.8 | AC |
| **4** | **KKS8410BR** | 5.25 | BE | 12.8 | BD | 2.14 | A | 0.15 | CD | 2.29 | CD | 1.3 | AB | 42.5 | BD | 32.7 | CE |
| **5** | **PAN5A_182** | 5.29 | BD | 12.8 | BD | 0.02 | C | 0.22 | CD | 0.24 | EF | 1.1 | AC | 42.0 | DE | 36.8 | AD |
| **6** | **IMP52_12R** | 5.34 | BC | 12.4 | CE | 0.05 | BC | 0.05 | D | 0.10 | F | 1.3 | AB | 42.6 | BC | 29.3 | E |
| **7** | **BG3292** | 5.27 | BD | 12.4 | CE | 0.08 | BC | 0.46 | CD | 0.54 | F | 1.4 | A | 45.5 | A | 32.3 | DE |
| **8** | **P2864WYR** | 5.20 | CE | 11.9 | E | 0.52 | AB | 0.62 | C | 1.13 | DE | 1.1 | C | 42.0 | DE | 39.3 | AB |
| **9** | **DKC78_79BR** | 5.39 | B | 13.0 | AC | 0.19 | BC | 16.48 | B | 16.67 | BC | 1.2 | AC | 42.3 | CD | 36.5 | AD |
| **10** | **LS8539B** | 5.28 | BD | 13.5 | A | 0.07 | BC | 22.11 | AB | 22.19 | AB | 1.2 | AC | 42.00 | DE | 35.8 | AD |
| **11** | **IMP52_11R** | 5.07 | EF | 13.0 | AC | 0.10 | BC | 26.02 | AB | 26.11 | AB | 1.3 | AB | 42.6 | BC | 32.4 | DE |
| **12** | **US9777** | 4.96 | F | 13.0 | AC | 0.07 | BC | 71.35 | A | 71.42 | A | 1.3 | AC | 42.3 | BD | 34.2 | BE |
| **13** | **US9711** | 4.97 | F | 12.9 | AC | 0.08 | BC | 27.22 | AB | 27.30 | AB | 1.3 | AC | 42.9 | B | 34.4 | BD |
| **14** | **KKS4581BR** | 5.20 | CE | 13.1 | AB | 0.02 | C | 30.10 | AB | 30.11 | AB | 1.2 | AC | 42.3 | CD | 36.9 | AD |
| **15** | **P1659W** | 5.16 | DE | 12.3 | DE | 0.08 | BC | 42.39 | AB | 42.47 | A | 1.2 | AC | 42.3 | CD | 34.4 | BE |
| | **Mean** | 5.3 | | 12.7 | | 0.3 | | 15.8 | | 16.1 | | 1.2 | | 42.5 | | 35.3 | |

Means followed by the same alphabetical letter in each column are not significantly different according to Fisher's LSD ($P \geq 0.05$).

*Mean recorded data followed by significant groupings based on log transformed mean.

**Table 12**. Pearson's correlation matrix of structural characteristics, physico-chemical properties and genetic responses with infection indicators for maize inbred lines evaluated during the 2017/18 season.

| | **Total fumonisins** | ***Fusarium verticillioides* target DNA** |
|---|---|---|
| **Pericarp thickness** | -0.18 | -0.02 |
| **100-kernel mass** | 0.51 | 0.57 |
| **Kernel hardness flour** | -0.46 | **-0.56 *** |
| **Moisture content** | **0.50 *** | 0.46 |
| **pH** | -0.18 | -0.11 |
| **Total nitrogen** | -0.08 | -0.08 |
| **Total carbon** | -0.19 | -0.13 |
| **C/N** | -0.09 | -0.02 |
| **Free phenolic acids** | 0.28 | 0.32 |
| **Bound phenolic acids** | -0.13 | -0.29 |
| **Total phenolic acids** | 0.01 | -0.15 |
| **Relative *PR1* expression** | **0.52 *** | **0.61 *** |
| **Relative *PR5* expression** | 0.54 | 0.69 |
| **Relative *peroxidase* expression** | **0.41 *** | **0.43 *** |
| **Total fumonisins** | 1.00 | **0.82 *** |
| ***Fusarium verticillioides* target DNA** | **0.82 *** | 1.00 |

**Bold *–** significant *P*-value with 95% confidence level ($p < 0.05$).

**Table 13**. Pearson's correlation matrix of structural characteristics and physico-chemical properties with infection indicators of maize cultivars evaluated in Potchefstroom during the 2016/17 season.

| | **FER severity** | **Total fumonisin (control)** | **Total fumonisin (inoculated)** | ***Fusarium verticillioides* target DNA (control)** | ***Fusarium verticillioides* target DNA (inoculated)** |
|---|---|---|---|---|---|
| **Silk length** | -0.09 | -0.06 | -0.22 | -0.19 | -0.03 |
| **Husk coverage (anthesis)** | 0.22 | -0.16 | 0.11 | 0.21 | -0.14 |
| **Husk coverage (harvest)** | **0.46 *** | -0.09 | 0.15 | 0.21 | -0.07 |
| **100-kernel mass** | 0.27 | -0.12 | -0.08 | -0.11 | 0.03 |
| **Kernel hardness kernels** | 0.29 | 0.11 | -0.05 | -0.09 | -0.20 |
| **Kernel hardness flour** | 0.09 | -0.05 | 0.08 | 0.07 | -0.13 |
| **Pericarp thickness** | 0.02 | -0.20 | -0.12 | -0.15 | -0.17 |
| **Moisture content** | 0.05 | 0.25 | **0.38 *** | 0.18 | 0.02 |
| **pH** | **-0.49 *** | -0.10 | -0.16 | -0.09 | -0.11 |
| **Free phenolic acids** | **0.32 *** | -0.17 | 0.00 | 0.28 | 0.06 |
| **Bound phenolic acids** | -0.04 | -0.13 | -0.09 | 0.10 | -0.01 |
| **Total phenolic acids** | **0.33 *** | -0.25 | -0.08 | 0.23 | 0.03 |

| | | | | | |
|---|---|---|---|---|---|
| **Total nitrogen** | **0.33 *** | 0.11 | **-0.35 *** | **-0.31 *** | 0.05 |
| **Total carbon** | 0.17 | -0.11 | -0.05 | -0.03 | -0.06 |
| **C/N** | 0.33 | -0.12 | **0.41 *** | **0.35 *** | -0.02 |
| **FER severity** | 1.00 | 0.23 | 0.10 | 0.23 | 0.05 |
| **Total fumonisin (control)** | 0.23 | 1.00 | 0.24 | 0.11 | **0.70 *** |
| **Total fumonisin (inoculated)** | 0.10 | 0.24 | 1.00 | **0.75 *** | -0.10 |
| ***Fusarium verticillioides* target DNA (control)** | 0.23 | 0.11 | **0.75 *** | 1.00 | -0.12 |
| ***Fusarium verticillioides* target DNA (inoculated)** | 0.05 | **0.70 *** | -0.10 | -0.12 | 1.00 |

**Bold *–** significant *P*-value with 95% confidence level ($p < 0.05$).

**Table 14**. Pearson's correlation matrix of structural characteristics, physico-chemical properties and genetic responses with infection indicators of maize cultivars evaluated in Vaalharts during the 2016/17 season.

| | **FER severity** | **Total fumonisin (control)** | **Total fumonisins (inoculated)** | ***Fusarium verticillioides* target DNA (control)** | ***Fusarium verticillioides* target DNA (inoculated)** |
|---|---|---|---|---|---|
| **Silk length** | -0.23 | -0.19 | -0.14 | -0.10 | -0.26 |
| **Husk coverage (anthesis)** | **0.45 *** | 0.15 | 0.23 | 0.29 | 0.15 |
| **Husk coverage (harvest)** | 0.17 | 0.13 | 0.10 | 0.20 | 0.08 |
| **100-kernel mass** | -0.07 | 0.05 | 0.01 | 0.08 | 0.05 |
| **Kernel hardness (kernels)** | -0.24 | -0.13 | -0.26 | -0.22 | -0.15 |
| **Kernel hardness (flour)** | 0.12 | 0.07 | 0.03 | 0.01 | 0.09 |
| **Pericarp thickness** | -0.09 | -0.05 | -0.05 | -0.05 | 0.07 |
| **Moisture content** | 0.01 | 0.10 | 0.09 | 0.11 | 0.06 |
| **pH** | **-0.73 *** | **-0.69 *** | **-0.72 *** | **-0.75 *** | **-0.58 *** |
| **Free phenolic acids** | -0.14 | -0.14 | -0.14 | -0.08 | -0.13 |
| **Bound phenolic acids** | **0.32 *** | 0.16 | **0.30 *** | 0.24 | 0.03 |
| **Total phenolic acids** | 0.27 | 0.10 | 0.16 | 0.10 | -0.01 |
| **Total nitrogen** | **0.34 *** | 0.29 | **0.30 *** | **0.32 *** | 0.25 |
| **Total carbon** | 0.01 | 0.05 | 0.13 | 0.11 | 0.00 |
| **C/N** | **-0.37 *** | **-0.41 *** | **-0.31 *** | **-0.36 *** | **-0.36 *** |
| **Relative *peroxidase* expression (control)** | 0.10 | **0.37 *** | 0.28 | 0.24 | **0.35 *** |

| | | | | | |
|---|---|---|---|---|---|
| **Relative *peroxidase* expression (inoculated)** | 0.18 | **0.39 *** | **0.40 *** | **0.36 *** | **0.34 *** |
| **Relative *PR5* expression (control)** | **0.46 *** | **0.59 *** | **0.59 *** | **0.53 *** | **0.60 *** |
| **Relative *PR5* expression (inoculated)** | **0.47 *** | **0.72 *** | **0.71 *** | **0.69 *** | **0.62 *** |
| **FER severity** | 1.00 | **0.58 *** | **0.69 *** | **0.63 *** | **0.55 *** |
| **Total fumonisin (control)** | **0.58 *** | 1.00 | **0.71 *** | **0.75 *** | **0.89 *** |
| **Total fumonisin (inoculated)** | **0.69 *** | **0.71 *** | 1.00 | **0.94 *** | **0.65 *** |
| ***Fusarium verticillioides* target DNA (control)** | **0.63 *** | **0.75 *** | **0.94 *** | 1.00 | **0.67 *** |
| ***Fusarium verticillioides* target DNA (inoculated)** | **0.55 *** | **0.89 *** | **0.65 *** | **0.67 *** | 1.00 |

**Bold *** – significant *P*-value with 95% confidence level ($p < 0.05$).

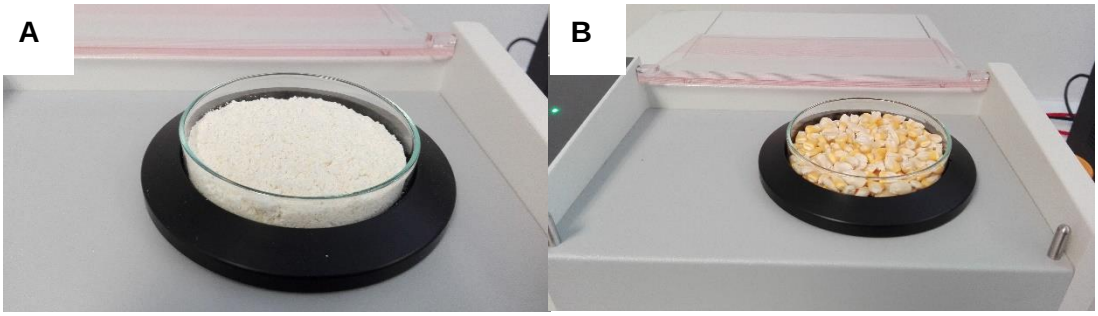


**Figure 1**. Near infrared (NIR) spectroscopy of (A) flour sample and (B) whole kernel sample. Samples were placed in a glass Petri plate and evaluated in the diffuse reflectance mode.

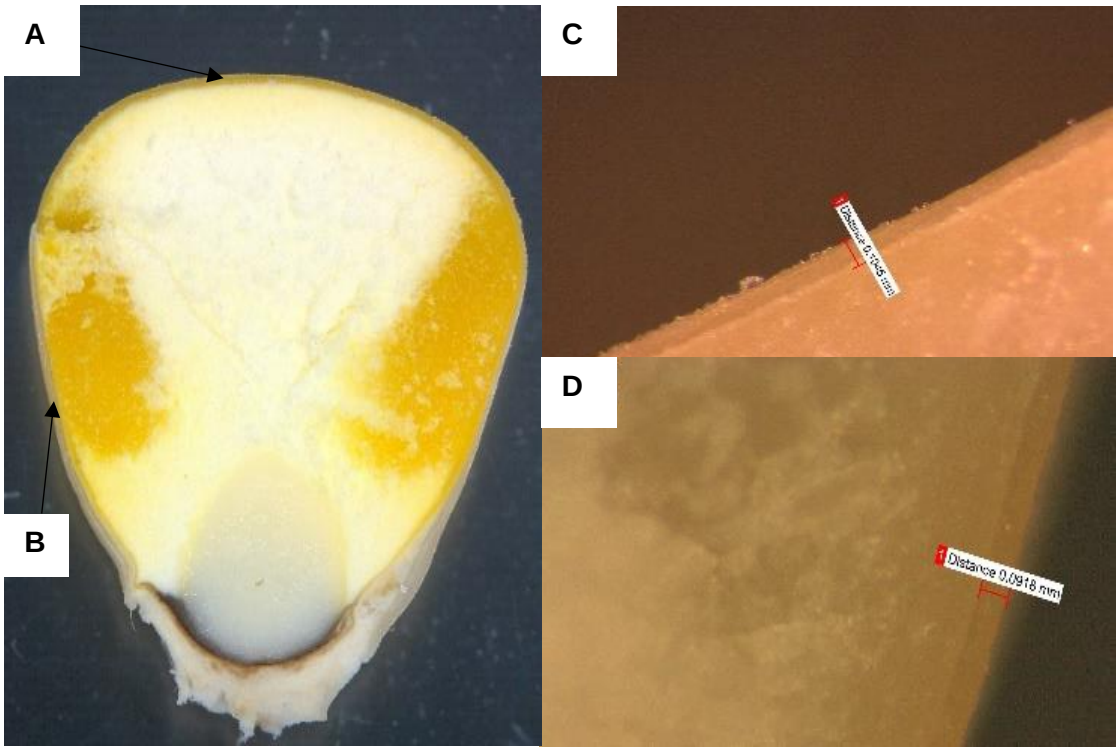


**Figure 2**. Longitudinal dissection of a maize kernel to assess kernel pericarp thickness. Two observations were recorded per kernel one at the (A) kernel top (dent; first observation) and one at the (B) middle to lower region (second observation). Kernel measurements were visualised on the LabScope mobile application (C-D).

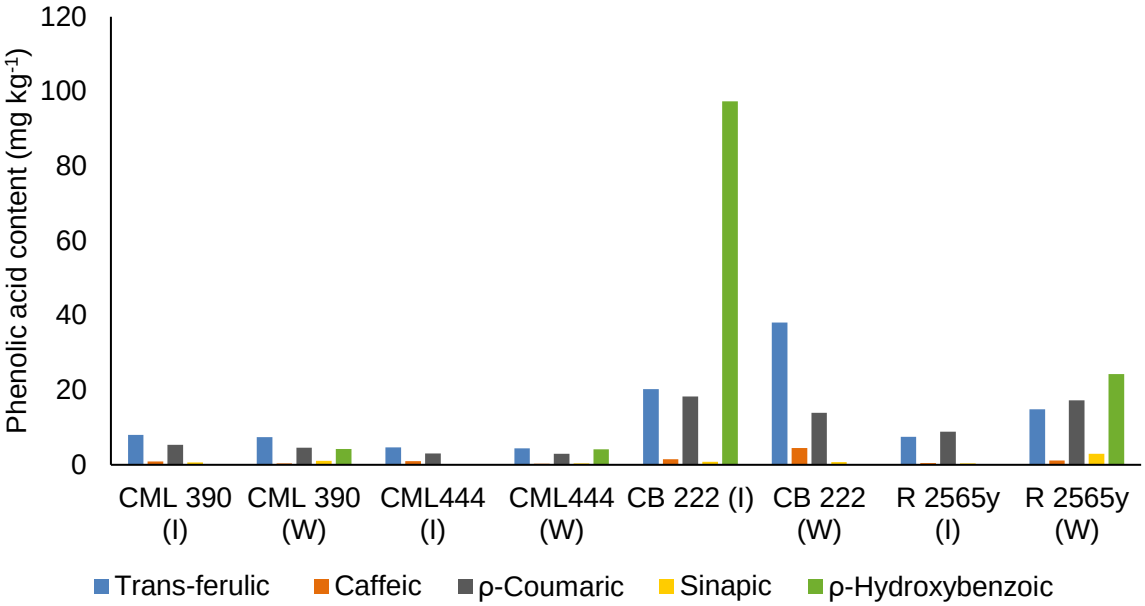


**Figure 3**. Free phenolic acids quantified in maize inbred lines. I – Inoculated (fungal-inoculated); W – Water-inoculated.

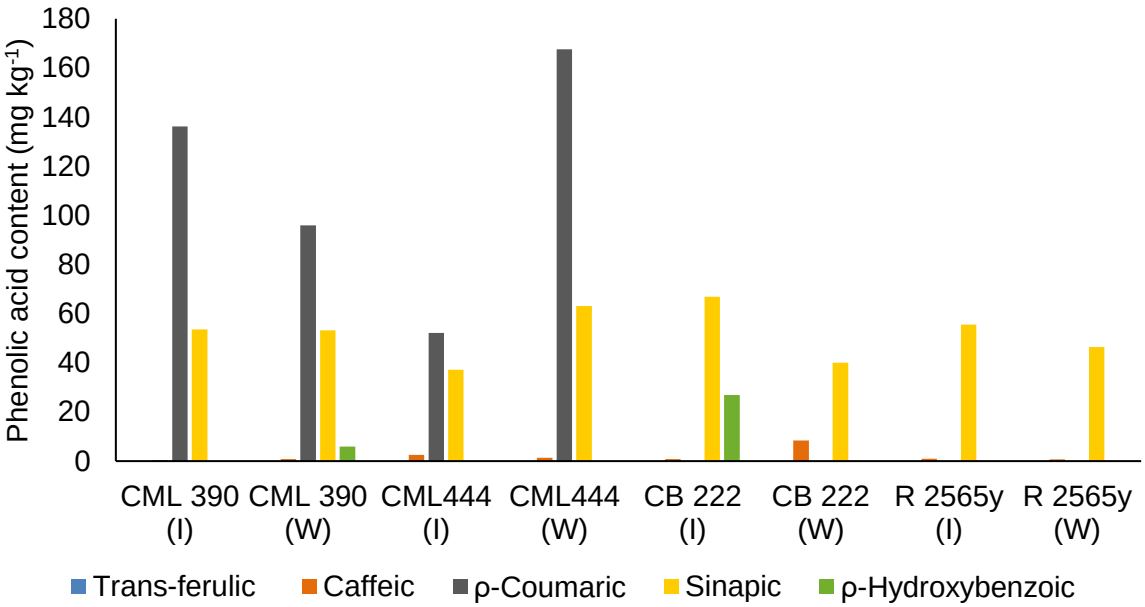


**Figure 4**. Bound phenolic acids quantified in maize inbred lines. I – Inoculated (fungal-inoculated); W – Water-inoculated.

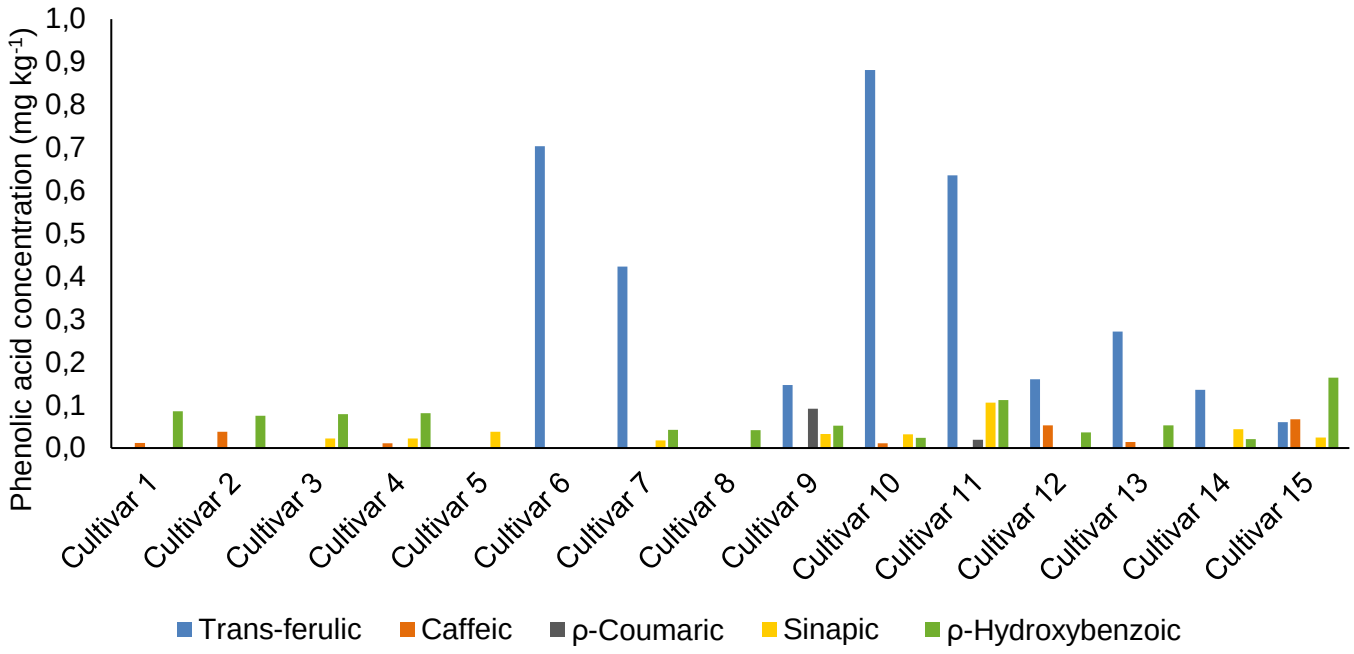


**Figure 5.** Free phenolic acids quantified in commercial maize cultivars inoculated with *Fusarium verticillioides* and grown in Potchefstroom.

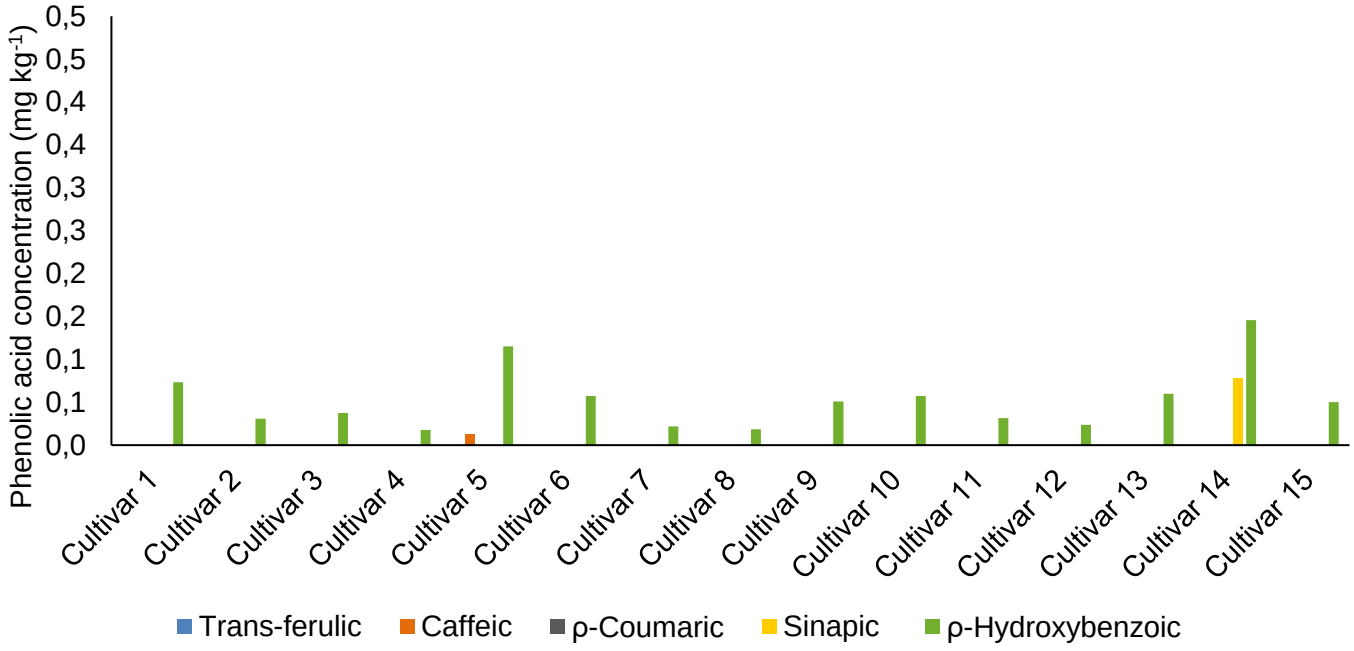


**Figure 6.** Bound phenolic acids quantified in commercial maize cultivars inoculated with *Fusarium verticillioides* and grown in Potchefstroom.

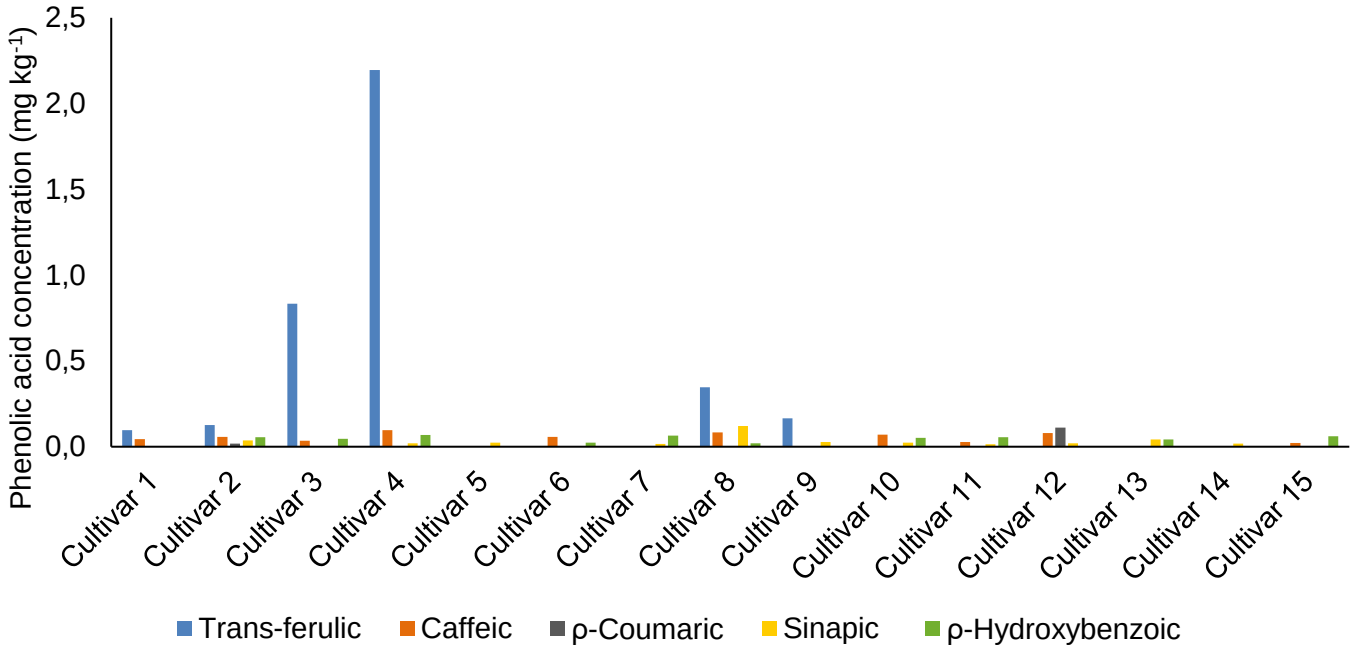


**Figure 7.** Free phenolic acids quantified in commercial maize cultivars inoculated with *Fusarium verticillioides* and grown in Vaalharts.

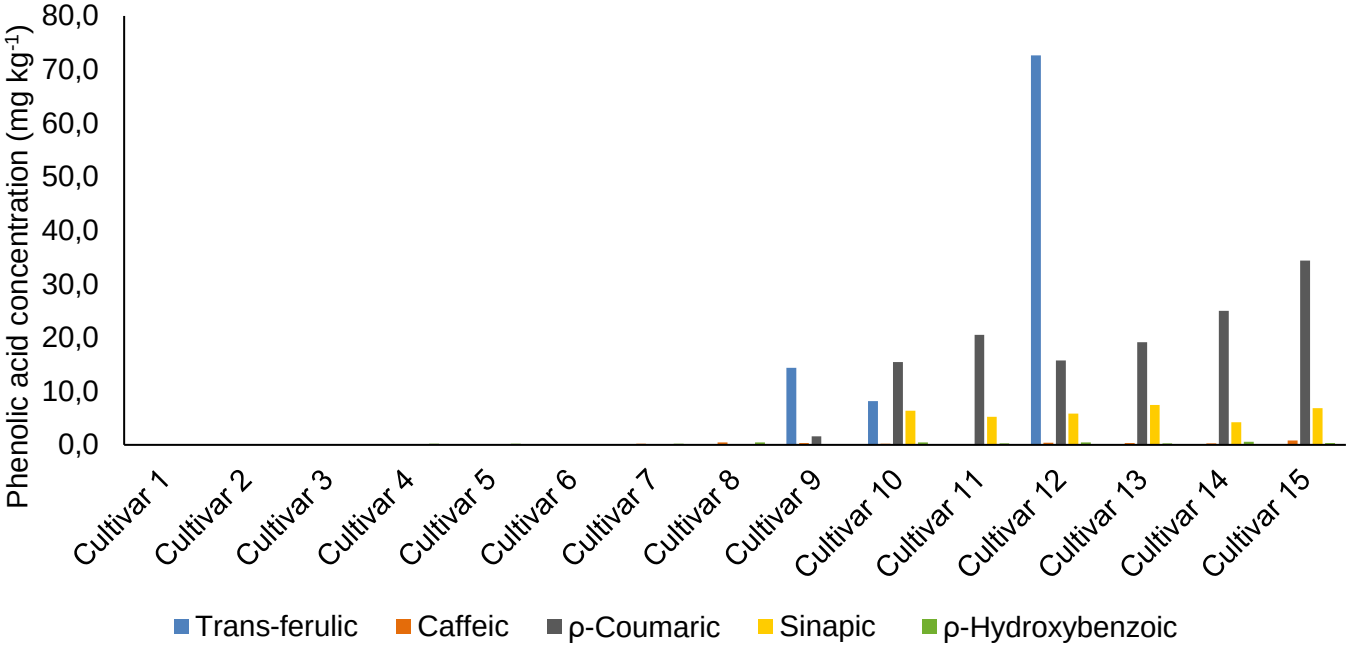


**Figure 8.** Bound phenolic acids quantified in commercial maize cultivars inoculated with *Fusarium verticillioides* and grown in Vaalharts.

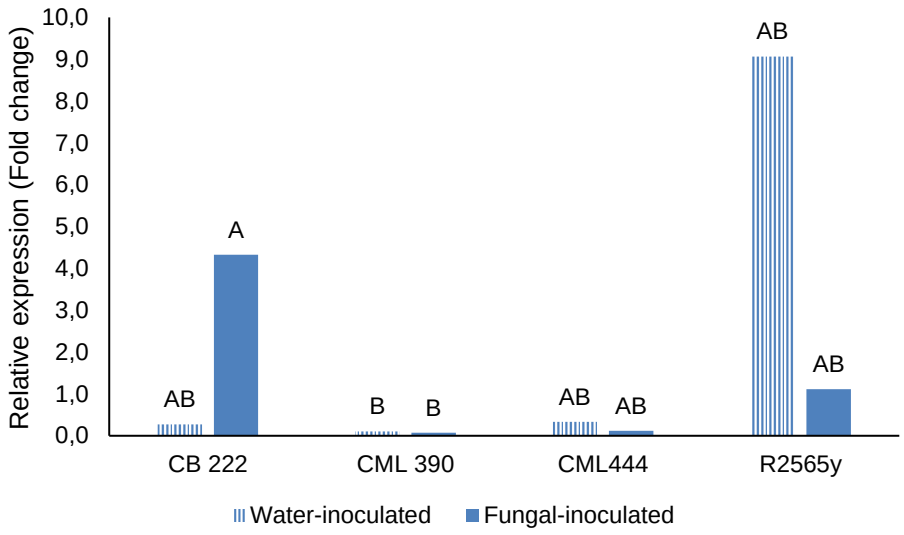


**Figure 9.** Relative expression of peroxidase gene in maize inbred lines grown under greenhouse conditions.

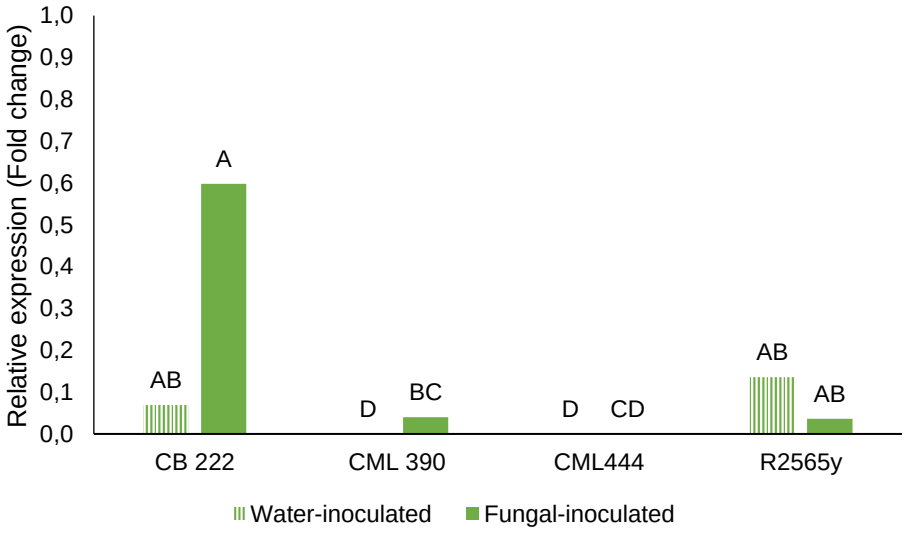


**Figure 10.** Relative expression of pathogenesis-related *1* (*PR1*) in maize inbred lines grown under greenhouse conditions.

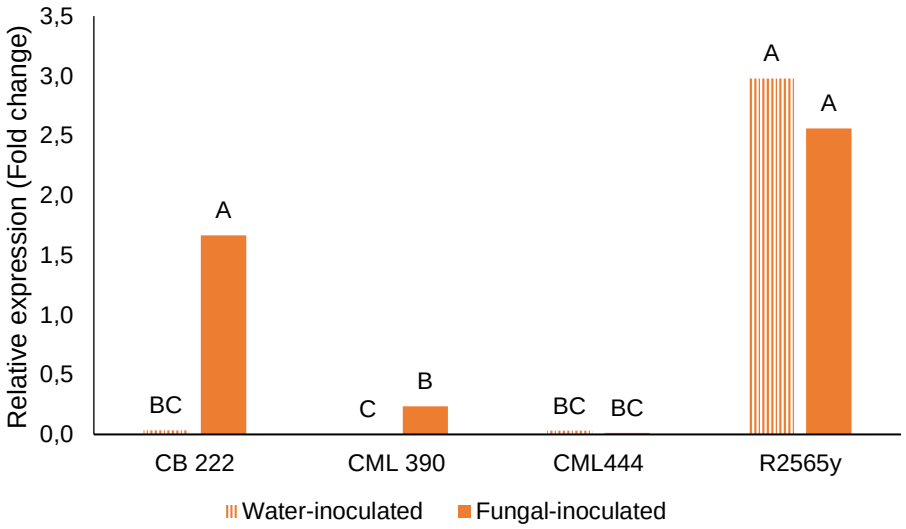


**Figure 11.** Expression of pathogenesis-related *5* (*PR5*) in maize inbred lines grown under greenhouse conditions.

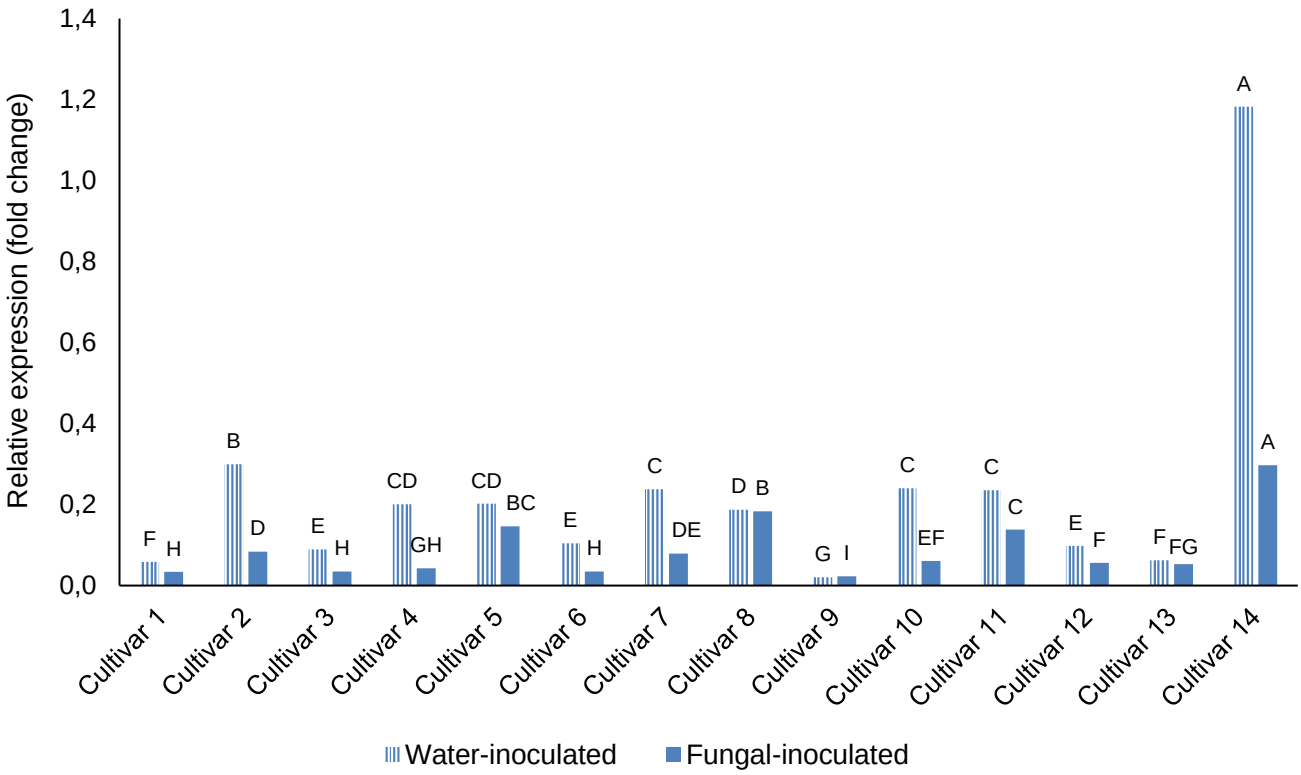


**Figure 12.** Expression of peroxidase gene in commercial maize cultivars grown in Vaalharts.

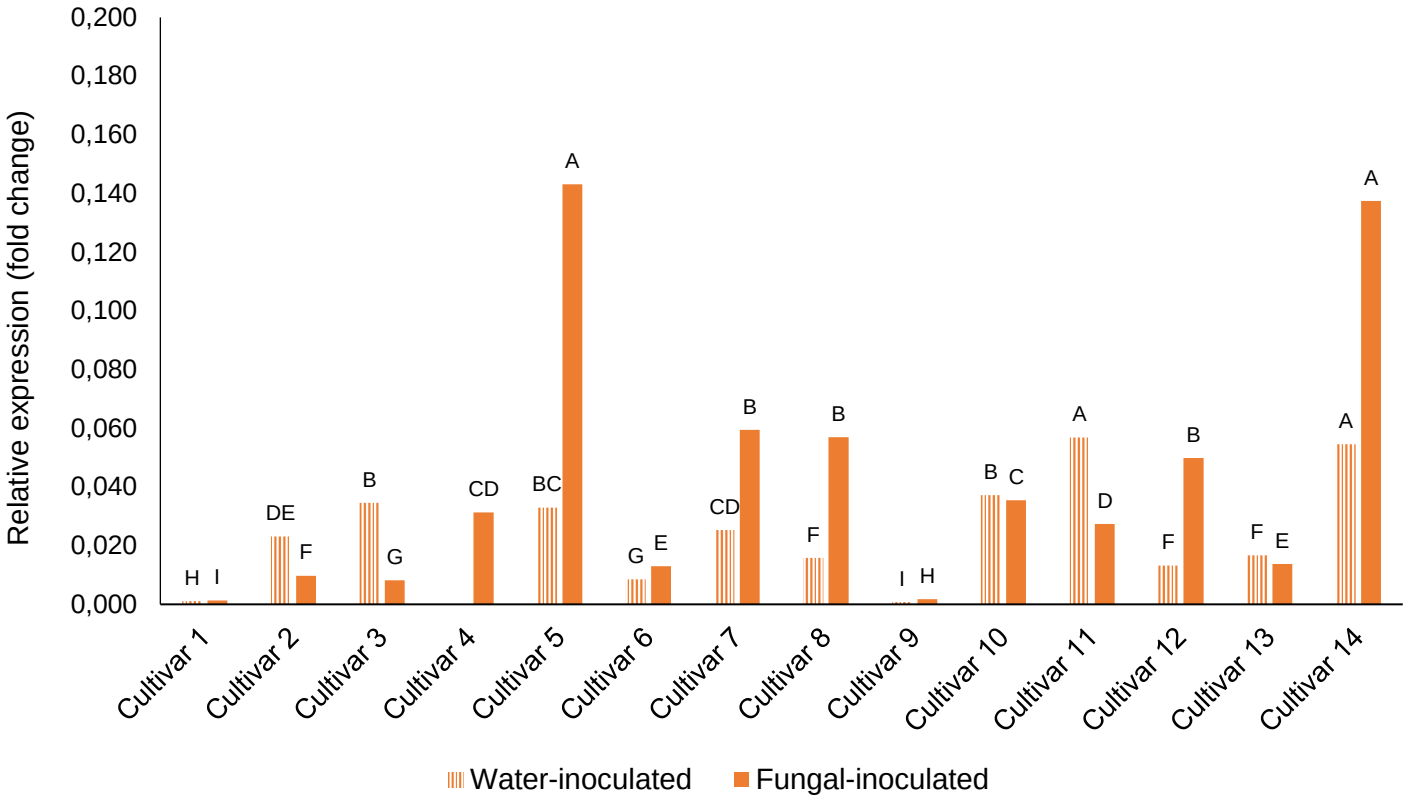


**Figure 13.** Relative expression of pathogenesis-related 5 (*PR5*) in commercial maize cultivars grown in Vaalharts.

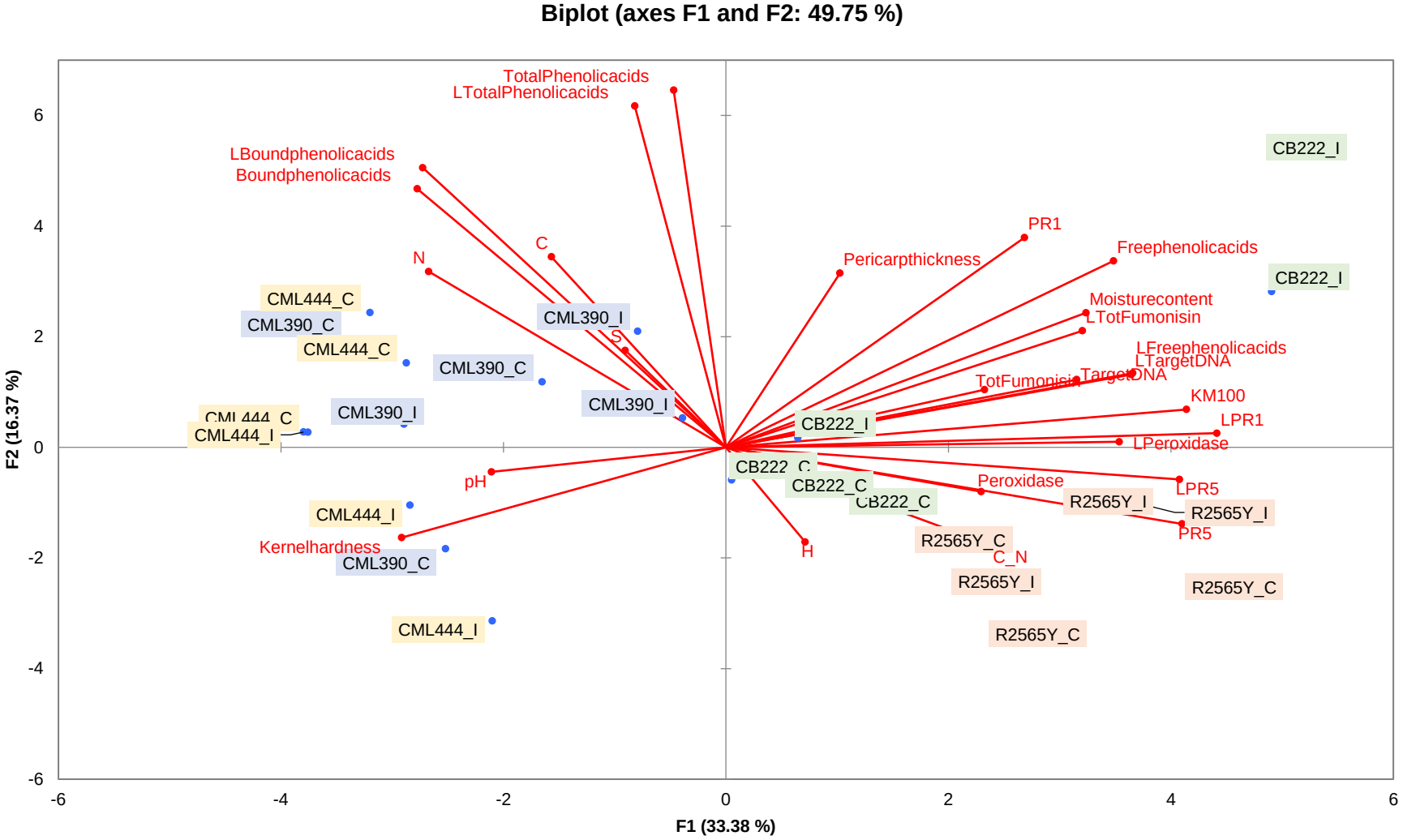


**Figure 14.** Principle component biplot of maize inbred lines showing all structural, physico-chemical and genetic characteristics evaluated.

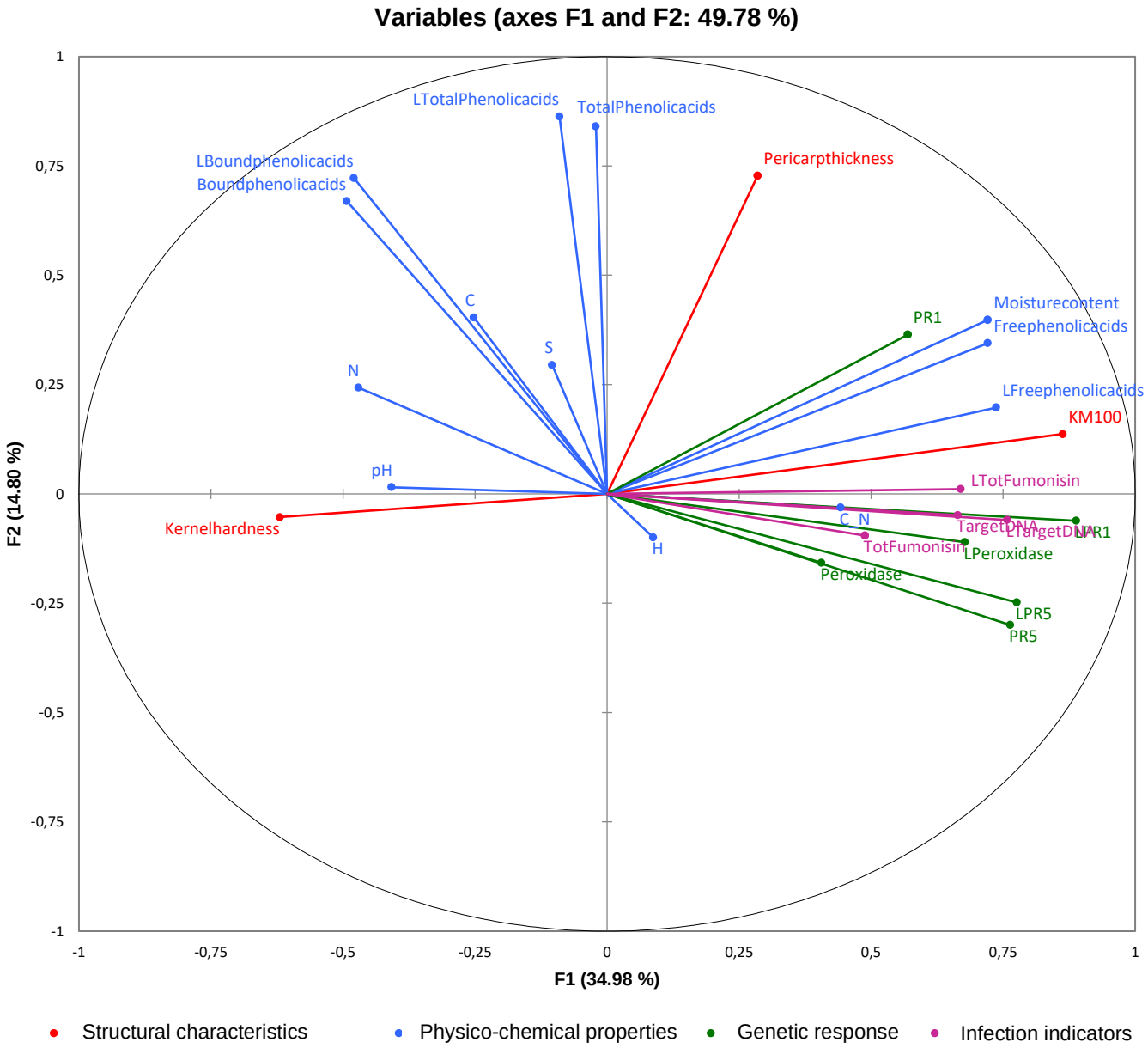


**Figure 15.** Multifactor biplot of inbred lines showing correlations between the different levels of resistance.

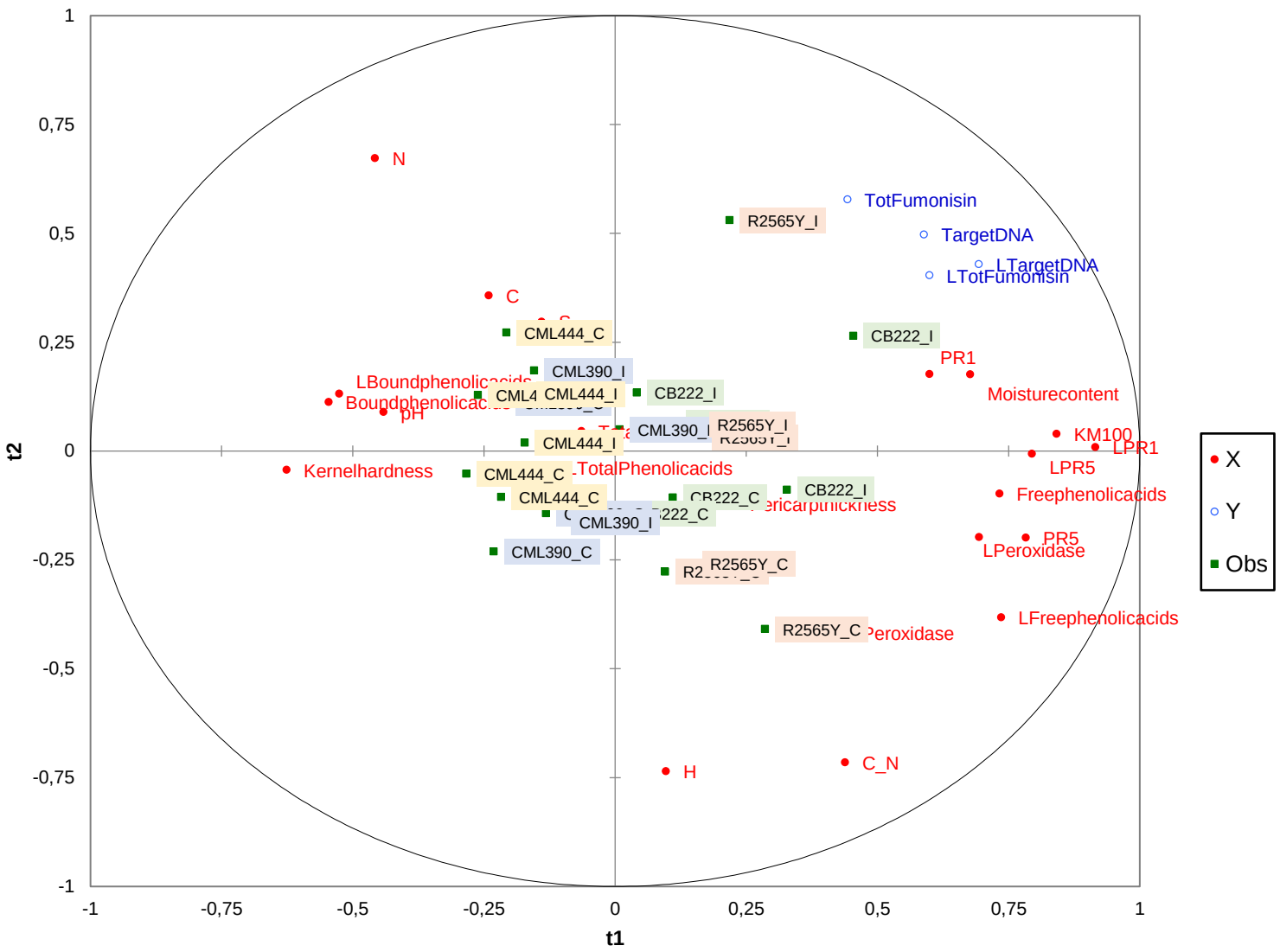


**Figure 16.** Partial least squared biplot of inbred lines indicating correlations of infection indicators with individual phenotypic, physico-chemical and genetic characteristics.

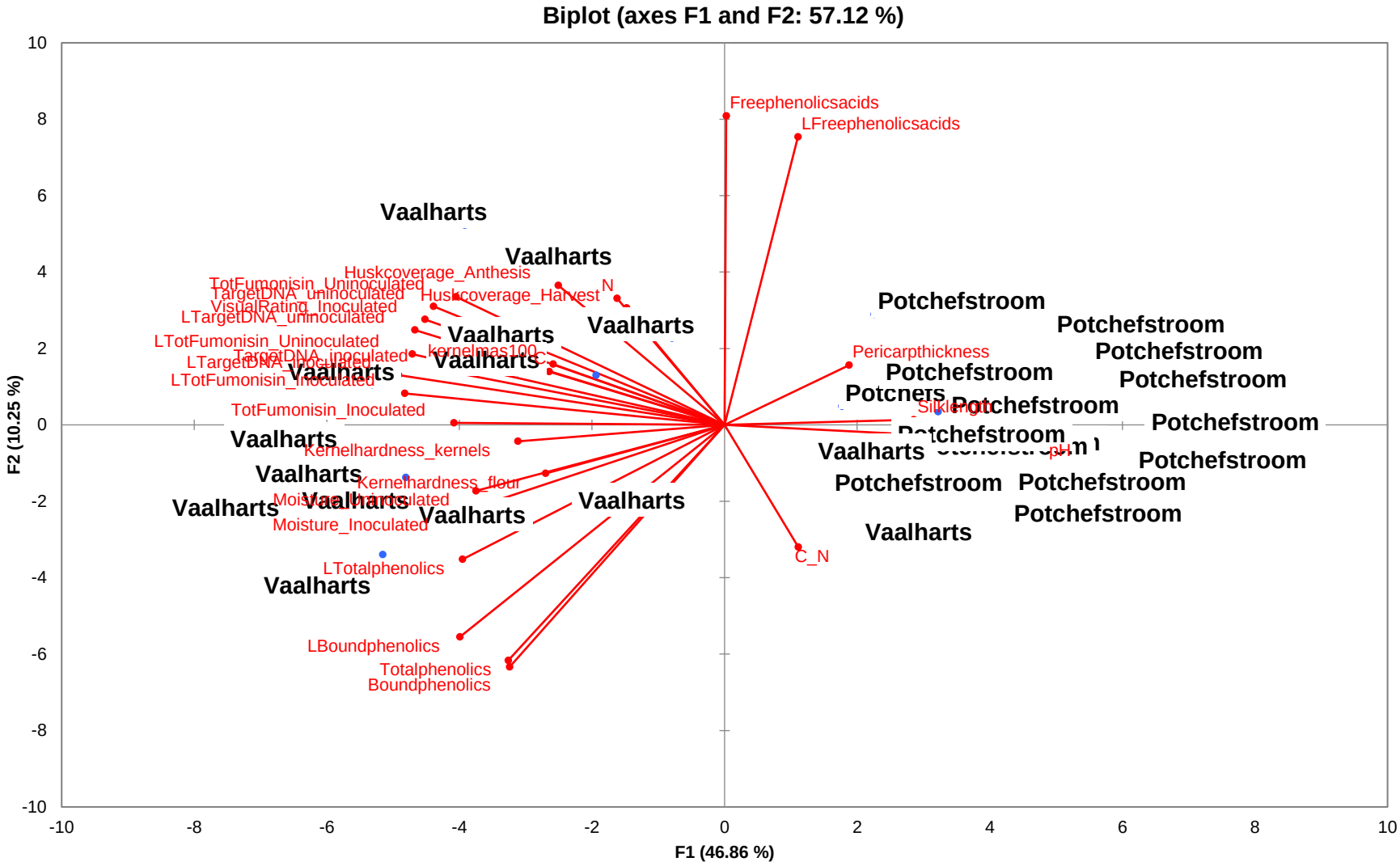


**Figure 17.** Principle component biplot of commercial maize cultivars showing all structural, physico-chemical and genetic characteristics evaluated.

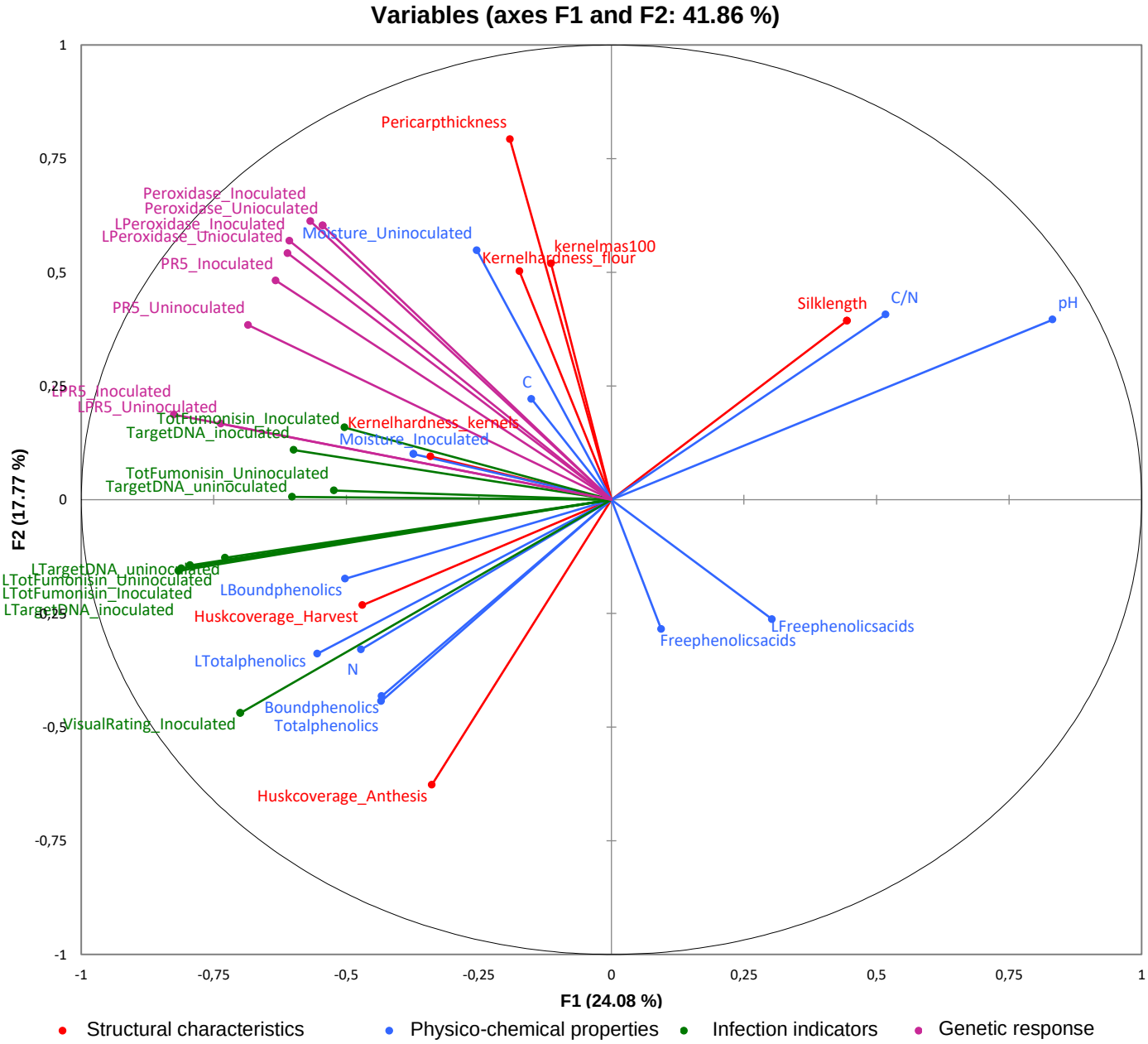




**Figure 18.** Multifactor biplot of commercial cultivars showing correlations between the different levels of resistance.

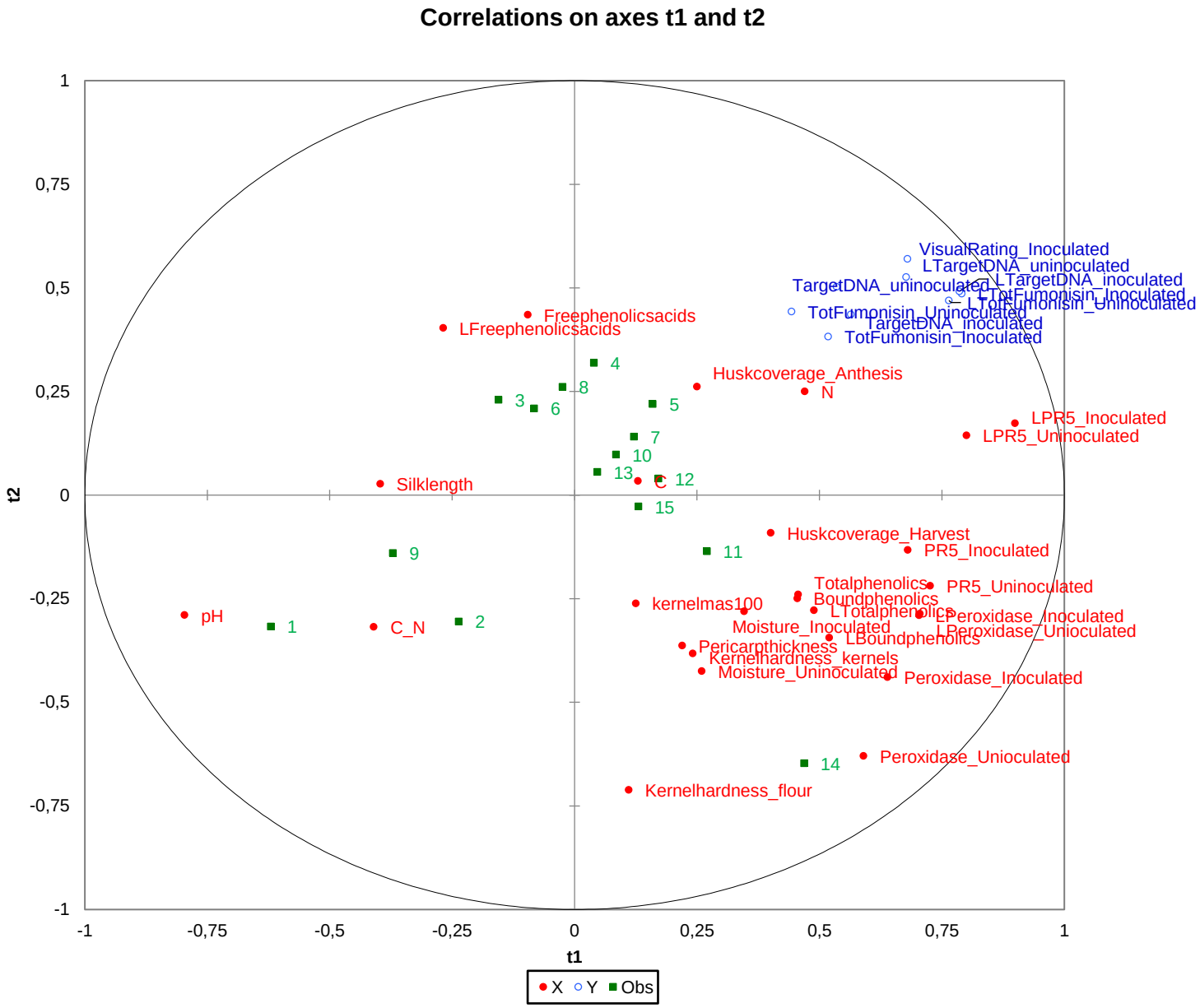




**Figure 19.** Partial least squared biplot of commercial cultivars indicating correlations of infection indicators with individual phenotypic, physico-chemical and genetic characteristics.